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## THE IMPORTANCE OF THE EPIDIDYMIS IN CASTRATION

CASTRATION EXPERIMENTS PERFORMED ON RATS BY  
MEANS OF ORCHIDOEPIDIDYMECTOMY AND ORCHI-  
ECTOMY, USING THE PROSTATE AS THE  
CRITERION OF RESPONSE

BY

P. I. TUOVINEN and R. POHJOLA

As is well known the urinary excretion of androgenic substances does not cease completely after castration, and it is generally assumed that the androgenic material found in the urine of castrated individuals originates from the adrenal cortex. Human male castrates excrete on an average from 1/5 to 1/3 of the amount of androgenic substances present in the urine of normal middleaged men. The excretion of 17-ketosteroids is also decreased after castration.

There have appeared in the literature a few reports of an increased secretion of hormones from the adrenal cortex after cessation of gonadal function in human subjects, but most of the investigations into the excretion of androgenic substances and 17-ketosteroids do not support this assumption. On the other hand, several animal experiments seem to indicate a compensatory hyperfunction of the adrenal cortex after castration.

The testis hormone is probably produced by the Leydig cells in the interstitial tissue of the testicles. Similar cells have, however, often been found outside the testicles. *Verocay* (1915) was the first to report the occurrence of interstitial cells in the surroundings of the testicles and along the hilar nerves in a case of cryptorchism in man, and *Berblinger* (1921) as well as *Kyrle* (1922) and *Priesel* (1924) found such cells in the epididymis in cases of cryptorchism and testicular atrophy in man and in the dog. *Cutore* (1922) found a gland, consisting of Leydig cells, in Henle's ampulla in the horse, donkey and mule. Formations which must be considered as accessory adrenals (Marchand's adrenals) were found by *Friedland* (1895), *Meyer* (1908) and *Brutschy* (1920) in the epididymis and along the spermatic blood vessels.

Since castration is followed by an increased production of hypophyseal gonadotrophic hormones, hypertrophy of extra-testicular Leydig cell tissue might result. This phenomenon could thus explain the results of animal experiments in which castration changes were found to be less marked than expected.

The physiological function of the epididymis is considered to be connected mainly with the transport, storing and ripening of the sperms. Further it probably resorbs poorly developed and over-ripe sperms (*Scherstén*, 1937). It is not generally believed to be of any importance in internal secretion (*Hotchkiss*, 1944), but few investigations dealing directly with this problem have been reported.

*Kornitzer & Lieben* (1924) established that the appearance of castration changes in the prostate cannot be prevented by feeding castrated animals with dried epididymis substance, but that it can be done with a testis substance. According to this the epididymis would have no androgenic function.

*Gallagher* (1928), however, found that by injecting capons with a dried preparation of bull's epididymis, the growth of the wattle was stimulated in the same way as by testis substance even though the effect was evidently less marked. Other organs were found to be ineffective.

On the basis of Gallagher's investigations *Lawless* (1931) performed control experiments on rats. In a series of 11 three weeks old rats he performed orchiectomy (hereafter abbreviated OE in our text) carefully avoiding any injury to the epididymis. Controls consisted of 10 rats of the same age castrated in the usual way, viz. by orchido-epididymectomy (OEE). He investigated the length and weight of the animals, the weight of the adrenals, thymus, liver, spleen, kidneys and brain. When eighty days old the animals were killed with chloroform and the results in the two series were compared. There were no appreciable differences, and from this fact the author concludes that the epididymis has no specific hormonal function. The accessory sexual glands are only casually mentioned; they were greatly atrophied in both series.

Among the investigations described above, the positive results obtained by *Gallagher* seem to be the only conclusive ones. One weakness of the experiments of *Kornitzer & Lieben* is the drying of the epididymis substance and the oral administration, which each separately may cause destruction of the hormones, or at least considerably lower their activity. In *Lawless'* investigation the experimental animals were too young to give reliable results.

A possible androgenic function of the epididymis still remains therefore to be investigated. The present investigation was undertaken because of the castration treatment which, in recent years, has been used in cases of metastasizing cancer of the prostate. The purpose of the treatment is to prevent as completely as possible the androgen secretion which stimulates the production of metastases. Both for cosmetic and psychological reasons the epididymises are not removed, when castration is performed in man, and only OE is performed. To preserve the natural appearance of the scrotum, *Lowsley & Kirwin* (1946) recommend removal only of the testis parenchyma, through a small incision of the tunica albuginea; when it is closed, a gradually organizing hematoma replaces the testis parenchyma, the result being deceptively natural. Thus two aims are in evident contrast to one another.

Since we know how highly sensitive the accessory sexual glands, particularly the prostate and the seminal vesicle, are to a cessation of the hormonal influence, we have chosen the prostate for a hormone test. We refer here to the wellknown investigations of *Moore et al.* (1930).

The purpose of the present investigation was to try to establish:

1) Whether there are any differences between orchietomy and orchidoepididymectomy, in the degenerative changes or in the period of development of such changes in the prostate of the rat?

2) Do these differences persist, or do they disappear as the effect of castration continues?

3) Is the possible androgenic influence of the epididymis a specific function characteristic of this organ, or does it depend on some external factors?

## MATERIAL AND TECHNIQUE

A total of 50 albino rats were used for the experiments. The age of the rats varied between 2 and 11 months. There were 17 2-month-old rats. Since rats were difficult to obtain at the time of the investigation castration could be performed on groups consisting of 4 to 8 rats only, and even then the experimental animals were not always of the same age. Castration was done under ether anesthesia and with the usual aseptic precautions. After castration the animals were kept under the same conditions as before and fed with the usual standard rations. They were not given the opportunity to mate.

The rats were killed by ether inhalation 1 to 12 weeks after castration. The prostate was immediately removed and fixed in 10 per cent formalin.

A summary of the experiments is given in Table 1.

Table 1.

Time since castration (weeks)	Number of rats		
	OEE	OE	Total
1	3	2	5
2	4	4	8
4	2	4	6
5	3	2	5
6	4	4	8
7	4	3	7
8	3	1	4
9	1	1	2
10	2	1	3
12	1	1	2
	27	23	50

Summary of the experiments (OEE = orchidopididymectomy; OE = orchiectomy).

## RESULTS

### THE EFFECT OF CASTRATION

#### *The interstitial connective tissue.*

In rats older than 3 months there is, after OEE an increase in the amount of the connective tissue of the prostate gland even after one week. After 4 weeks the increase is marked, reaching its maximum after 6 to 8 weeks. At that time the tissue is also very loose. From the ninth week onwards the connective tissue seems to be firmer again. After 10 to 12 weeks there is no appreciable increase and the connective tissue is now very firm.

After OE there is no increase in the amount of the connective tissue until after the fourth week. One week later, the increase is evident, reaching its maximum 7 to 8 weeks after castration. At that time the connective tissue is at its loosest. After the ninth week it seems to get firmer again and to become fibrotic. 10 to 12 weeks after castration the results are the same as in cases of OEE.

In rats less than 3 months old there is an increase in the amount of the connective tissue only 7 weeks after castration.

*The result of castration* manifests itself in an enlargement

and simultaneous loosening of the connective tissue. In cases of OEE this occurs within one week whereas in cases of OE the changes do not appear until after 4 weeks. The maximum in both groups is reached after 8 weeks. Then there is an evident decrease in the connective tissue which, at the same time, becomes firmer again, and these changes appear simultaneously in both groups. After 12 weeks the connective tissue is fairly normal in quantity, being, however, firmer than normal.

In rats less than 3 months old the enlargement sets in only after the seventh week. There are no differences with regard to the different operative methods.

### *Epithelium.*

In rats more than 3 months old the epithelium of the prostate gland atrophies gradually and evenly both after OEE and OE. The epithelial cells are shrunken and crowded together by the pressure of the enlarged connective tissue. This causes a folding of the epithelium into two or three layers. This phenomenon is first noticed in the small acini after 4 weeks. At the same time the cuticular border becomes fragmented and disappears in places. The cytoplasm becomes clearer and vacuolated. The nuclei still stain heavily and some of them also show pyknosis. In some nuclei, however, granules of chromatin occur.

5 to 7 weeks after castration these changes gradually become more marked. The epithelium becomes detached from its basement membrane, and begins to degenerate. Ample desquamation occurs in the lumina. The nuclei are, as a rule, already granular and there are even numerous unstained nuclei.

After 8 weeks the degeneration seems to have reached its maximum. Thereafter the epithelium begins somehow to regenerate. It becomes regular again. The cuticular border reappears in places. The nuclei become round but still show granules of chromatin. There are in fact some which stain quite normally, but they are very rare.

10 to 12 weeks castrates present an almost similar picture to 8 weeks castrates. *The regeneration does not seem to continue.*

When comparing the development of degeneration after OEE and OE it seems as if it were, during the first weeks, somewhat slower in cases of OE than in cases of OEE. The differences are, however, so slight that they cannot be considered significant. From the fourth week onwards the degeneration is equal in both groups.

Rats less than 3 months old form an obvious exception to the description given above. The degeneration is considerably slower and no difference in the cases of the different operative methods can be noticed. Not until 6 weeks after castration do changes in the epithelium become noticeable. The epithelium remains as a rule normal and regular but shows some folding. The cells and nuclei generally remain large but the nuclei stain less heavily. The cuticular border becomes fragmented and disappears in places.

In castrated rats more than 3 months old the epithelium degenerates evenly until the eighth week when the maximum is reached. Thereafter the epithelium again becomes more regular, but after 12 weeks the nuclei are still granular. *Degeneration during the first two weeks seems to be a little slower in cases of OE than in cases of OEE. The difference is, however, so slight that it cannot be considered significant.* In rats less than 3 months old degeneration does not commence until the sixth week, and the changes are slight.

## THE OCCURRENCE OF SECRETION

### *Intracellular secretory granules.*

Heidenhain's iron hematoxylin staining: In cases of OEE and OE of rats more than 3 months old a heavily staining supranuclear secretory granule can generally be seen until one week after castration. In many places these granules form a dark band around the lumen. After 2 weeks there are hardly any bands of this kind left, but small supranuclear granules



still occur in large numbers. With the gradual development of the degeneration the granules decrease in number but are still found, although sparsely, in seven weeks castrates. After 7 weeks no granules can be found. In rats less than 3 months old there are very few supranuclear granules one week after castration. After two weeks they have disappeared completely.

Altmann-Kull staining: In cases of OEE and OE of rats more than 3 months old a supranuclear secretory granule staining red can generally be seen until one week after castration. In many places these granules form a band around the lumen. After 2 weeks there are still isolated granules but the bands have disappeared. After 3 weeks the cytoplasm still stains a light red but no red granules can be noticed. They are in many places replaced by blue granules. After 5 weeks no violet colour change can be observed in the cytoplasm and the bluish granules have also disappeared.

In rats less than 3 months old the cytoplasm stains a light violet colour one or two weeks after castration. Granules do not occur.

In rats more than 3 months old, intracellular secretory granules still occur in great numbers 2 weeks after castration. Later they decrease rapidly. In Heidenhain's staining a few may still occur 6 weeks after castration. In Altmann-Kull's staining the last granules can be seen 2 weeks after castration. After 4 weeks the cytoplasm still stains a light red but later the reddish colour does not appear any more.

In rats less than 3 months old, supranuclear granules occur sparsely in Heidenhain's staining 1 and 2 weeks after castration. In Altmann-Kull's staining no granules can be seen but the cytoplasm still stains a light violet 2 weeks after castration.

*With regard to the intracellular secretory granules no difference can be observed between the effect of OEE and OE.*

#### *Extracellular secretory granules.*

Heidenhain's staining: In cases of OEE and OE of rats more than 3 months old, heavily-staining secretory granules

occur here and there on the outer surfaces of the epithelial cells towards the lumen one week after castration. After 4 weeks some of these are still visible but not after 5 weeks.

In rats less than 3 months old these granules do not occur.

Altmann-Kull's staining: In cases of OEE and OE of rats more than 3 months old there are a few extracellular secretory granules which stain red 1 and 2 weeks after castration. They cannot be found later.

In rats less than 3 months old extracellular granules do not appear.

Extracellular secretory granules occur after castration only in rats of 3 months or older. There are very few granules which disappear in Heidenhain's staining 4 weeks and in Altmann-Kull staining 2 weeks after castration. *No difference can be noticed between the effects of OEE and OE.*

#### *Comparison of results in cases of OEE and OE.*

Differences in the results can only be noticed in rats older than 3 months and even then the differences are slight. They are most clearly seen during the course of the degeneration of the interstitial connective tissue. In cases of OEE, degeneration as shown by increase of the connective tissue can be observed as early as one week after castration, whereas this change does not occur until 4 weeks after castration in cases of OE. With regard to the epithelium a somewhat delayed degeneration during the first two weeks may be present, but this is uncertain. There are no differences with regard to the secretion. *The differences cannot be considered significant.*

## DISCUSSION AND CONCLUSIONS

*Moore et al.* (1930) in their investigation dealt separately with the anterior lobe of the prostate and they believed that castration changes were slower in the anterior lobe than in other lobes. Although it has a different appearance it reacts, in our opinion, in exactly the same way as the other lobes, and we have therefore dealt with the prostate as a whole. The light

supranuclear area in the epithelial cells to which these authors refer and whose importance they particularly stress, is apparently an intracellular secretory granule, as it stains lightly with hematoxylin- van Gieson. Since it shows up much better after Heidenhain's and Altmann-Kull's staining, we have used these methods.

The Golgi apparatus and the mitochondria do not, according to the above authors, react readily to castration and we have therefore ignored them.

The castration changes found by us do, on the whole, agree with those reported with regard to the intracellular secretory granule. According to these authors the granules disappear by the fifth day after castration whereas we found them as late as 6 weeks after castration. The explanation will probably be found in the different staining methods.

With Altmann-Kull staining the intracellular secretory granule becomes blue immediately before its final disappearance during the fourth week. This is probably due to a change in the acidity of the secretion as a result of castration. A similar phenomenon was observed in the seminal vesicle of the rat (*v. Lanz, 1931*).

Although the function of the epididymis is comparatively independent, it is, none-the-less controlled to a certain degree by the testicles. After removal of the testicles it shrinks and its secretion ceases. The influence of the testicle is, however, not brought about by circulating hormones since, both in cases of unilaterally aplastic testicle and of unilateral atrophy, the epididymis on the same side is quiescent in spite of the fact that it is active on the other side. Therefore the effect upon the epididymis must be transmitted by some other means. The following have been suggested: the nerve pathways common to the testicle and the epididymis, the common circulatory system, and the passing sperm (*v. Lanz, 1926*). In a consideration of the results of castration, the nerve pathway and the common circulatory system are of no importance, since the testicles are removed. But there is, of course, always sperm left in the epididymis. It has been asserted that by injecting

castrated cocks with sperm collected from the epididymis, the growth of the wattle was stimulated. This has, however, not been confirmed. It is possible that Leydig's cells secrete testis hormone into the seminiferous tubules and that the hormone thus gets into the sperm. On the other hand, experiments have been made by injecting young female rats, rabbits, and guinea-pigs with ejaculated human sperm. These injections had no effect at all whereas injections of testosterone caused an evident enlargement of the uterus and the clitoris (*Bacsich et al.*, 1945). According to these investigations, which, it is true, are of very little significance, there is testis hormone in the sperm contained in the epididymis, whereas testis hormone is not to be found in ejaculated sperm. Hence it must be resorbed by the organism somewhere along the passage of the sperm. There is every reason to believe that the resorption takes place in the epididymis, as its epithelial gland cells show, in addition to the usual streaming towards the lumen, a streaming in the basal direction which suggests a resorbing activity (*Nassonov*, 1927). *Patzelt* (1946) in his textbook of pathology, mentions the possibility of a resorption by the epididymis of testis hormone from the passing sperm.

The possible hormonal effect of the epididymis may thus, according to the literature quoted above, be explained in two ways:

- 1) The epididymis contains cells secreting testis hormone, viz. Leydig's cells or adrenal cortical cells which function like an independent gland under the control of the anterior lobe of the hypophysis.

- 2) The hormonal effect is due to the capacity of the epididymis to resorb testis hormone from the sperm contained in it, and to transmit it to the general circulation.

In the former case removal of the testicles should in no way lessen the hormonal secretion of the epididymis. More probably an increase in hormonal secretion would result from the increased amount of gonadotrophin that follows castration. In the latter case the hormonal effect of the epididymis will, of course, soon cease since new sperm is not produced.

*Our investigation therefore supports the latter assumption.* The final result was the same both in cases of OEE and OE; at most a slightly slower degeneration during the first weeks could be observed in cases of OE.

*The main conclusions* of our investigation are therefore as follows:

1) Both OEE and OE produce degenerative changes in the prostate; in rats less than 3 months old the course of these changes is evidently slower than in adult rats.

2) The degeneration reaches its maximum 8 weeks after castration, after which there is a partial regeneration in about the tenth week.

3) Slight secretion continues after castration until the seventh week both after OEE and OE.

4) The degeneration of the interstitial connective tissue in the first four weeks and of the epithelium in the first two weeks seems to be somewhat slower after OE than after OEE. The differences are however, slight and difficult to estimate, and they cannot be considered significant.

5) The investigation did not show anything that would support the hypothesis of a specific hormonal effect of the epididymis. However, the sperm stored in the epididymis at the time of castration may be of some importance. The testis hormone contained in it is evidently resorbed into the organism and may, for a short time, delay the occurrence of castration changes.

6) With regard to the final result it was found that there was no difference if the testis was removed alone or together with the epididymis.

## SUMMARY

The purpose of the investigation was to determine the degenerative changes in the prostate of the rat after orchido-epididymectomy (OEE) and orchiectomy (OE). These operations were performed on 50 rats and the animals were killed from one to twelve weeks after the operation.

The castration changes were observed in the fibro-muscular stroma, in the alveolar epithelium and in the intra- and extra-cellular granules of the epithelium. It was established that both OEE and OE caused the degenerative changes in the prostate that have been described in the literature. The degeneration reached its maximum 8 weeks after castration, after which there was some regeneration up to the tenth week. After both operations secretion continued until the seventh week. During the first four weeks the degeneration of the fibro-muscular tissue seemed to be somewhat slower in cases of OE than in cases of OEE. The possibility can therefore not be ruled out that the epididymis actively resorbs testis hormone from the passing sperm and thus acquires some androgenic significance.

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*Fig. 1.*

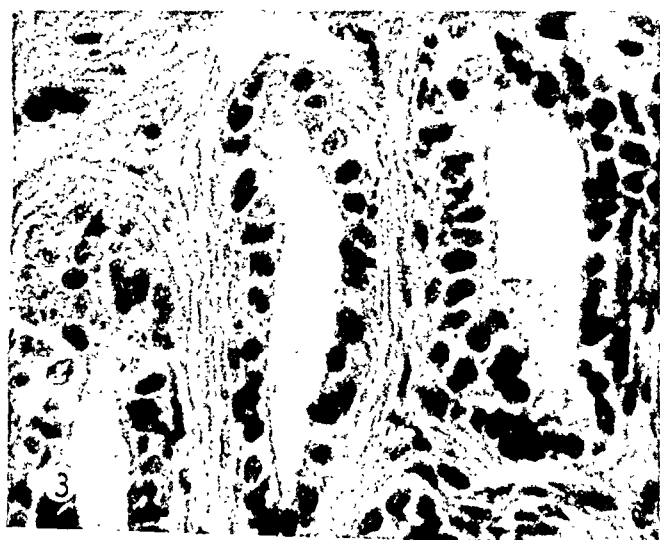
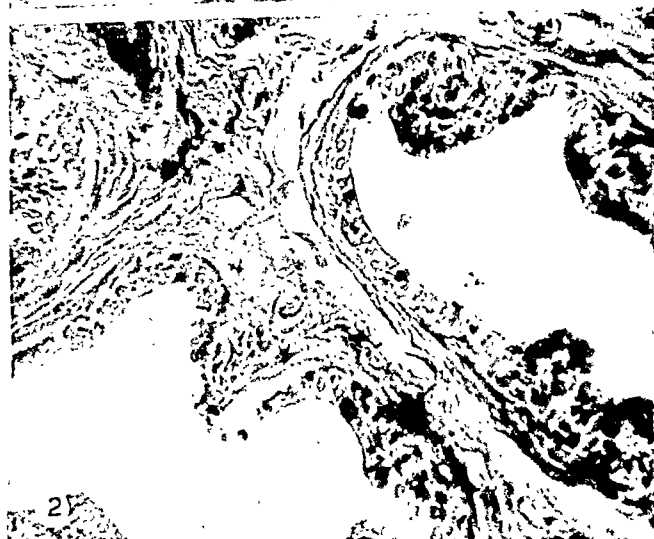
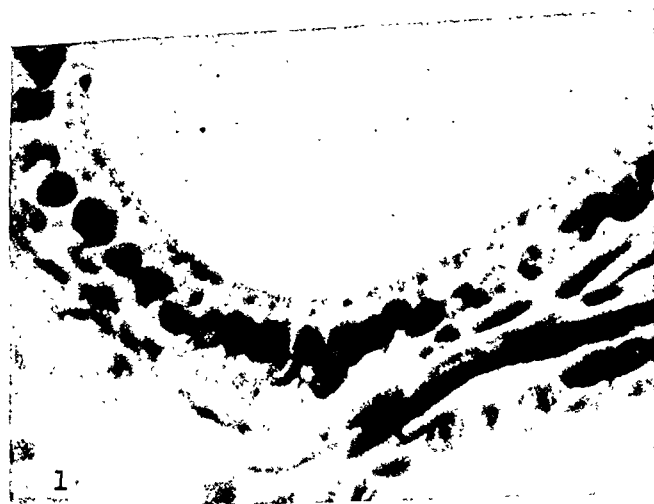
OEE on a rat of 7 months (No 6). Epithelium of the prostate 4 weeks after operation.  $\times 900$ . Heidenhain's iron hematoxylin. The nuclei heavily stained. Intracellular secretory granules in a regular row.

*Fig. 2.*

OE on a rat of 7 months (No 14). Epithelium of the prostate 6 weeks after operation.  $\times 300$ . Heidenhain's iron hematoxylin. No granules visible. The nuclei spotted, lightly stained.

*Fig. 3.*

OEE on a rat of 6 months (No 28). Epithelium of the prostate 10 weeks after operation.  $\times 550$ . Heidenhain's iron hematoxylin. Epithelium shows some regeneration. Most of the nuclei spotted or lightly stained, but some heavily stained.







*Fig. 4.*

OEE on a rat of 7 months (No 11). Epithelium of the prostate 2 weeks after operation.  $\times 300$ . Altmann-Kull. Secretory granules visible on the surface of the epithelial cells.

*Fig. 5.*

OEE on a rat of 2 months (No 57). Epithelium of the prostate 2 weeks after operation.  $\times 550$ . Heidenhain's iron hematoxylin. The cuticular border clearly visible. The nuclei have commenced to stain lightly.

Travail de la Clinique Thérapeutique Universitaire de Genève.  
(Prof. G. Bickel)

EFFET DE L'HORMONE ADRÉNOCORTICO-  
TROPE SUR LA MORPHOLOGIE DU CORTEX  
SURRÉNALIEN, SON CONTENU EN ACIDE  
ASCORBIQUE ET EN ESTERS DE CHOLÉSTEROL  
CHEZ LE RAT NORMAL

PAR

PIERRE DUCOMMUN et RENÉ S. MACH

Les fonctions et la grandeur des surrénales dépendent de l'activité hypophysaire. Ces relations connues depuis longtemps sont mises en évidence par des faits cliniques et expérimentaux: l'atrophie corticosurrénaliennne et les signes cliniques d'insuffisance corticale apparaissent fréquemment dans les syndromes d'hypopituitarisme antérieur (*Simmonds, Cushing, Moss, Robertson, Sarason, Soffer, Swann, Bickel*). Expérimentalement cette dépendance est devenue une notion classique depuis les travaux de *Smith* (1930), *Evans* (1932) et *Collip* (1933), qui les premiers, ont démontré l'atrophie sur-

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Nous sommes très reconnaissants au Dr. *H. Selye*, Directeur de l'Institut de Médecine et de Chirurgie expérimentales de l'Université de Montréal (Canada) de nous avoir permis de terminer et de contrôler ces recherches dans ses laboratoires.

Nous remercions vivement le Prof. *E. Rutishauser*, Directeur de l'Institut Pathologique de Genève pour les conseils qu'il nous a donnés.

L'hormone hypophysaire corticotrope que nous avons utilisée nous a été fournie par la maison Organon. Nous lui adressons ainsi qu'à son Directeur scientifique, le Dr. *Tausk*, nos remerciements.

rénalienne chez les animaux hypophysectomisés. Cette atrophie est limitée au cortex et n'atteint jamais le médullaire (*Hous-say*). Elle est corrigée par l'injection d'extraits hypophysaires ou par une greffe de la glande. Déjà à cette époque, on a pu mettre en évidence l'apparition de l'atrophie au niveau de la zone réticulée (atrophie et picnose nucléaire) suivie de l'extension du processus à la zone fasciculée (dégénérescence aréolaire). D'autres modifications plus discrètes ont été décrites: ratatinement de l'appareil de Golgi (*Reese*), modifications dans la répartition du plasmalogène (*Tonutti*).

Comme l'activité hypophysaire surrénalotrope n'est dirigée que vers le cortex, *Swann* a proposé le terme de facteur adrénocorticotrope ou d'hormone adrénocorticotrope (ACTH). Cette hormone a été isolée de l'hypophyse du porc où elle se trouve en plus grande quantité que dans celle du mouton ou celle du boeuf (*Astwood & Tyslowitz*, 1942). On a pu la déceler en très faible quantité dans le sérum de jument gravide (*Golla & Reiss*, 1942) et dans l'urine de femme (*Blumenthal*, 1945).

L'ACTH actuellement employée dans des buts cliniques et expérimentaux peut être préparée à partir de l'hypophyse de porc selon la technique de *Sayers, White & Long* (1943), ou à partir de l'hypophyse de mouton suivant la méthode de *Li et al.* (1942, 1943).

Ses propriétés sont connues et ont été rassemblées par *Li & Evans* (1948): point isoélectrique 4,65 à 4,80 (*Li, Evans & Simpson*, 1943; *Sayers, White & Long*, 1943), poids moléculaire environ 20.000; contenu: 46,3 % de C, 5,89 % de H, 2,3 % de S, 4,5 % tyrosine, 1 % tryptophane, 1,93 % méthionine, 7,19 % cystine (*Li*); pas d'hydrates de carbone, pas de phosphore, pas de cystéine; soluble dans l'eau, stable à la chaleur (*Li, Evans & Simpson*, 1943; *Noble & Collip*, 1941).

A l'état normal, le cortex surrénalien est riche en lipides, en cholestérol et en acide ascorbique.

L'action de ACTH sur le cortex peut être divisée en deux phases:

a) *période de décharge*, qui suit immédiatement l'administration d'une dose unique, caractérisée histologiquement par une

diminution de la soudanophilie et chimiquement, par une chute du taux des esters de cholestérol qui atteint environ la moitié de sa valeur initiale 3 à 6 heures après l'injection (Sayers, Sayers, Fry, White & Long, 1944; Sayers, Sayers, Liang & Long, 1946). Le taux de l'acide ascorbique suit les mêmes variations, mais sa chute est plus brutale et plus précoce (environ 60 % après une heure).

Cette action apparaît aussi bien chez les animaux hypophysectomisés que chez les animaux intacts (Sayers). Ces modifications sont les mêmes que celles produites lors d'un »stress« avec cette différence que dans ce cas, l'hypophysectomie prévient la chute du cholestérol et de l'acide ascorbique (Levin, 1945; Ludewig & Chanutin, 1946).

b) *période de recharge*. Si l'on ne donne qu'une seule injection d'ACTH, le taux de cholestérol redevient normal en 24 heures, alors que celui de l'acide ascorbique dépasse les taux initiaux dès la douzième heure.

L'administration régulière de petites doses d'ACTH détermine, après la décharge initiale, une augmentation lente et progressive du poids et du volume de la surrénale, qui se traduisent histologiquement par une surcharge en substance soudanophile et chimiquement par une élévation du taux des esters de cholestérol. Ces variations sont les témoins d'une activité corticale augmentée. Les mêmes constatations sont valables pour les »stress« légers et prolongés.

Une très forte dose d'ACTH donnée régulièrement détermine une vidange totale du cholestérol et de l'acide ascorbique, qui ne varieront pas jusqu'à la mort de l'animal. Cet épuisement est accompagné d'une hypertrophie corticale qui est proportionnelle au temps qui s'écoule entre le début des injections et la mort.

Ces phénomènes sont en tous points comparables aux notions de réactions d'alarme et de syndrome général d'adaptation de Selye (1946), qui suivent n'importe quel agent alarmant agissant dans les mêmes conditions.

Le but de notre travail a été le suivant:

1. Déterminer chimiquement les variations des esters de cholestérol et de l'acide ascorbique surrénalien après une dose unique d'ACTH.
2. Déterminer histologiquement ces variations par les colorations au Soudan et nitrate d'argent acide.

L'ACTH nous a été fournie par la fabrique Organon. Son étude clinique, au moyen du test de *Thorn*, a été faite par l'un de nous (*Mach*) à la Clinique Thérapeutique de Genève.

## PROTOCOLE EXPERIMENTAL

Rats mâles noirs et blancs, pesant entre 150 et 220 grammes. Régime préliminaire standard (eau, purina).

A jeun durant la nuit précédant l'expérience et durant les 24 heures de l'expérience; eau à volonté.

Injection de 2 mg ACTH pure Organon, pour 100 gr de poids d'animal.

Sacrification des animaux aux heures suivantes: 0, 1, 3, 6, 10, 24 (saignée).

Détermination esters de cholestérol selon *Ludewig & Chanutin* (1946).

Détermination acide ascorbique selon *Roe & Kuether* (1943).

Un groupe témoin, recevant 2 mg d'albumine, sacrifié aux mêmes heures que les animaux d'expérience.

Poids frais des surrénales.

Coloration des surrénales au Soudan.

Coloration des surrénales au nitrate d'argent acide selon la méthode *Leblond* et Ecole de Harvard modifiée.

## EXAMENS CHIMIQUES

Les résultats des déterminations de cholestérol et d'acide ascorbique sont résumés dans le tableau 1 et la fig. 1.

*Tableau 1* représentant les variations des taux de l'acide ascorbique, des esters de cholestérol, et du poids de la surrénale. Les résultats donnés sur ce tableau sont calculés sur une

Tableau 1.

Intervalle administra- tion ACTH et mort.	Poids surrénales		Cholestérol			Acide ascorbique		
	Nb. de Rats	mg. p. cent gr.	Nb. de Rats	mg. % poids frais	%	Nb. de Rats	mg. % P. frais	%
0 heures	10	7,63	6	4,2	100	6	3,66	100
1 "	10	8,69	8	3,1	73,8	8	2,07	56,5
3 "	10	7,40	8	2,3	54,7	8	2,08	55,8
6 "	10	8,90	8	3,9	92,8	8	3,02	82,5
10 "	10	11,4	8	4,5	107,1	8	3,70	101
24 "	10	11,3	8	4,2	100	8	3,79	103,5

moyenne de 8 à 10 animaux, sacrifiés à chaque heure, en employant l'analyse statistique.

(Les dosages ont été faits par Madame *Fischer*, chimiste, à l'Institut Pathologique de Genève).

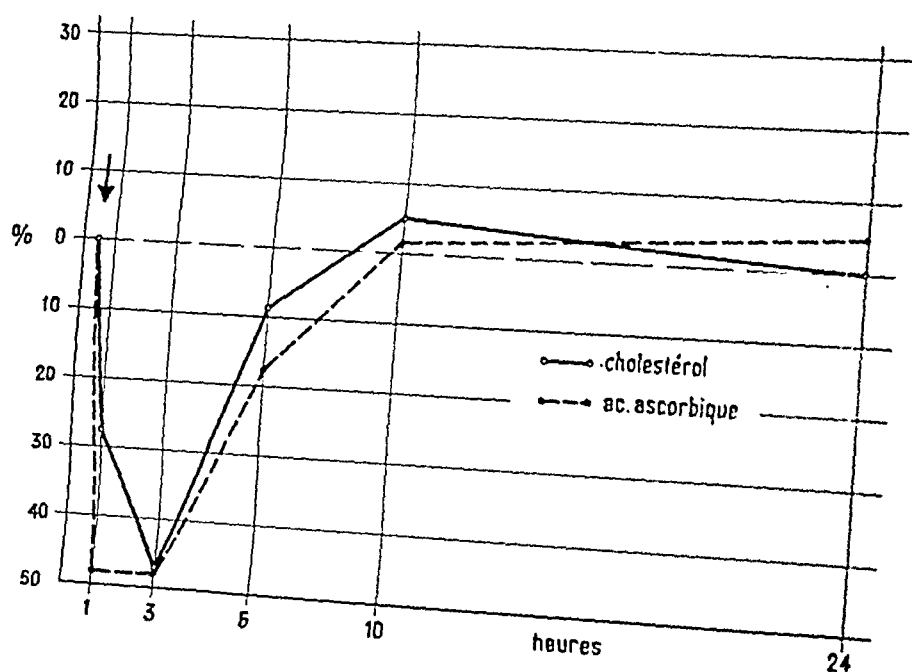


Fig. 1.

Variations du taux du cholestérol et de l'acide ascorbique du cortex de la surrénale du rat, après injection de 2 mg. d'ACTH.

## EXAMENS HISTOLOGIQUES

1. *Etude de la soudanophilie* (voir fig. 2).

- a) Une heure après l'injection d'ACTH, la substance soudanophile diminue considérablement, comparativement au taux observé chez les témoins absolus ou chez les animaux injectés avec une albumine inactive.

La zone glomérulaire est plus riche que les autres zones corticales.

La zone fasciculaire moyenne en contient un peu plus. Macroscopiquement, les surrénales sont un peu plus brunes que normalement.

- b) 3 heures après l'injection, la vidange est quasi totale, plus marquée dans les zones fasciculaire et réticulaire que dans la glomérulée.

Macroscopiquement, les surrénales sont grandes et brunes.

- c) 6 heures après l'injection, la substance soudanophile est en augmentation dans les zones fasciculaire et réticulaire, et a atteint un taux normal dans la zone glomérulaire.
- d) 10 heures après l'injection, le cortex a repris son aspect normal; on ne peut plus apprécier par les variations tinctorielles une différence dans le taux soudanophile cortical.
- e) 24 heures plus tard, la surrénale est normale ou plus riche.

Nous arrivons aux mêmes résultats par l'examen des coupes non colorées, au moyen de la lumière polarisée.

2. *Etude de l'acide ascorbique* (voir fig. 3).

De l'examen des coupes des surrénales colorées par notre méthode, nous tirons les conclusions suivantes:

- a) Avant l'injection d'ACTH, l'acide ascorbique est précipité sous forme de petits grains noirs irréguliers de grandeur, irrégulièrement répartis dans les cellules et semblant prendre la place du chondriome (*Leblond*).

La capsule et la zone glomérulaire en sont dépourvues. Les zones fasciculaire et réticulaire en sont riches (a) et il est très facile de distinguer la limite entre le cortex et la médullaire, par l'arrêt des granulations. Les ilots corticaux

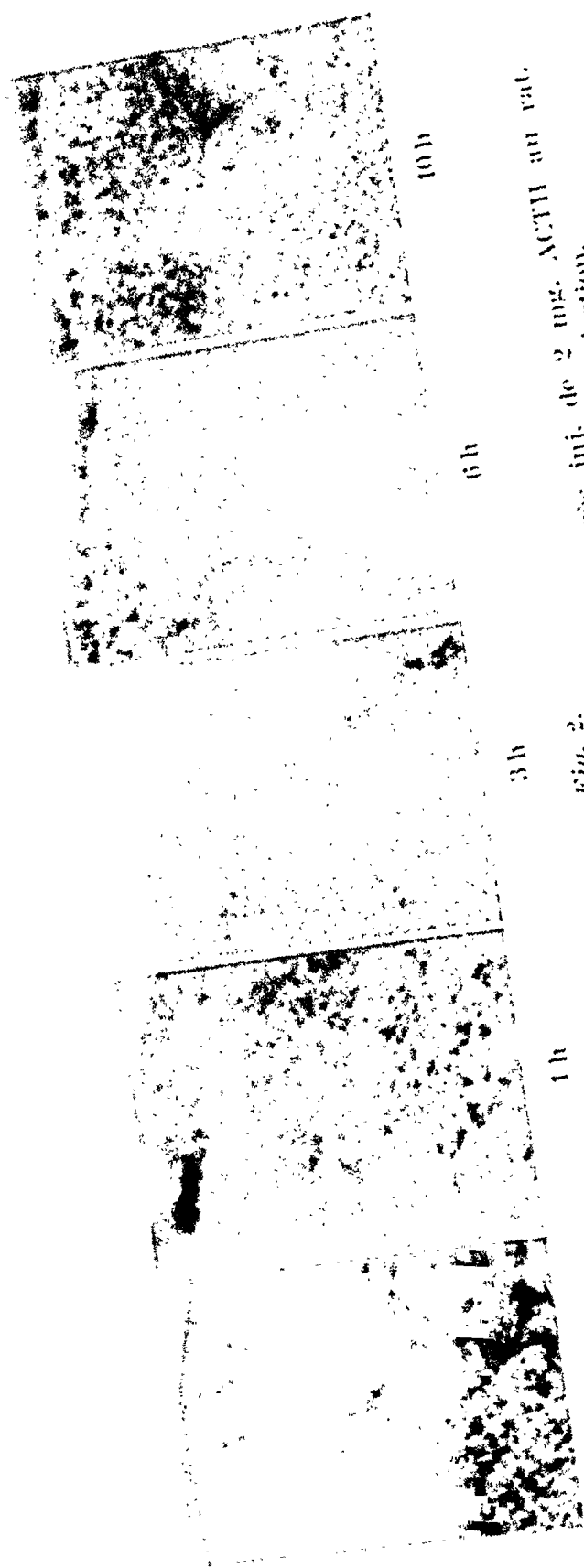
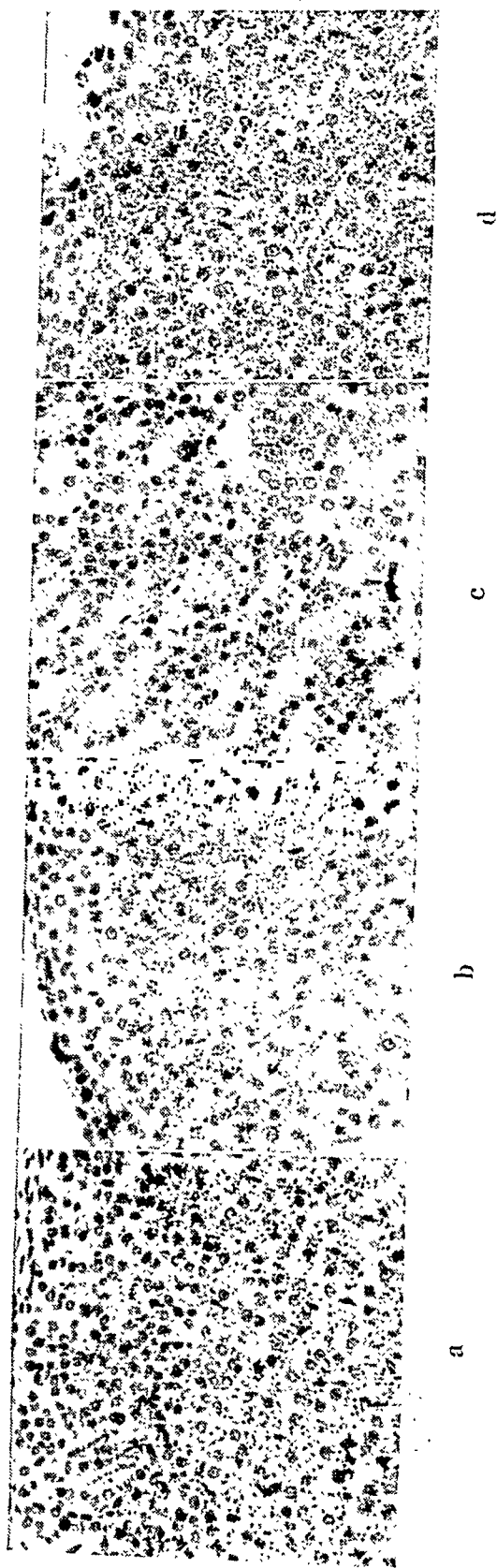


Fig. 2.

Variations de la substance soudanophile du cortex surrénal après inj. de 2 mg. ACTH au rat. Microphotos: N (avant), 1 heure, 3 h., 6 h., et 10 h. après l'injection.







*Fig. 3.*

Variations de l'ac. ascorbique du cortex après inj. de 2 mg. d'ACTH.  
Microphotos: (a) avant, (b) 1 heure, (c) 3 h., (d) 6 h. après l'injection.

intramédullaires se colorent de la même façon que le cortex. Dans quelques rares cellules médullaires, on trouve un précipité beaucoup plus fin que celui des cellules corticales.

Cette méthode de coloration n'est pas exempte d'artefacts, qui se produisent très facilement, si l'on ne contrôle pas rigoureusement les solutions et les temps de coloration.

- b) Une heure après l'injection d'ACTH, on observe une diminution globale de l'acide ascorbique de la zone fasciculaire (b). Il semble que la plus forte diminution porte sur la partie inférieure de la zone réticulaire. Les ilots corticaux intra-médullaires suivent les mêmes variations. Les cellules médullaires contenant le fin précipité de nitrate d'argent ne sont pas modifiées.
- c) 3 heures après l'injection, recharge nette de la zone réticulaire inférieure (c), peu de variations dans les zones réticulaire supérieure et fasciculaire. Pas de modifications de la zone glomérulée.
- d) 6 heures après l'injection, aspect comparable à celui des témoins absolus et à celui des contrôles injectés d'albumine inactive (d).

### EN RESUME

L'injection d'une dose unique d'ACTH (2 mg% gr. de poids de l'animal) détermine

morphologiquement:

Une augmentation du poids et du volume de la surrénale, avec une dégranulation visible macroscopiquement.

chimiquement:

1. Chute brutale mais transitoire du taux de l'acide ascorbique surrénalien.
2. Chute plus lente des esters de cholestérol.

histologiquement:

1. Diminution de la soudanophilie moins accentuée au niveau de la glomérulée que dans les autres zones corticales.
2. Diminution de l'acide ascorbique, surtout au niveau de la

zone inférieure réticulaire, la recharge se faisant principalement par la même zone.

Inactivité de la zone glomérulaire en regard du métabolisme de l'acide ascorbique.

Nos expériences démontrent que les modifications histologiques et chimiques sous l'effet de l'injection d'ACTH sont beaucoup moins prononcées dans la zone glomérulaire que dans les autres zones du cortex. Cette différence s'observe aussi bien pour la teneur en cholestérol qu'en acide ascorbique.

### SUMMARY

*Pierre Ducommun and René S. Mach: Effects of adrenocorticotrophic hormone (ACTH) on the morphology of the adrenal cortex, its content of ascorbic acid and of cholesterol ester in the normal rat.*

The injection of a single dose of ACTH (2 mg. per 100 gm. body weight) causes

#### *Morphologically:*

An increase in weight and volume of the suprarenals, with macroscopically visible degranulation.

#### *Chemically:*

1. A sharp but transitory fall in suprarenal ascorbic acid.
2. A slower fall in cholesterol esters.

#### *Histologically:*

1. Diminution of sudanophilia, less marked in the glomerular than in the other cortical zones.
2. Decrease in ascorbic acid, especially in the inferior reticular zone, through which accumulation of ascorbic acid chiefly takes place.

Inactivity of the glomerular zone as far as the metabolism of ascorbic acid is concerned.

Our experiments show that the histological and chemical modifications produced by ACTH are much less marked in the glomerular than in the other cortical zones. This difference applies both to the cholesterol and ascorbic acid content.

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From the Research Laboratories of the Teva Middle East  
Pharmaceutical and Chemical Works Ltd., Jerusalem.

## ANTAGONISM BETWEEN THE ADRENOTROPHIC HORMONE OF THE HYPOPHYSIS AND THE SEX HORMONES

BY

HANS HASE\*) and LEO SCHINDEL

Although the fundamental significance of the adrenal cortex in physiology and pathology has long been known, little was done to clarify the role of the adrenotrophic hormone of the pituitary in controlling the hormonal function of the adrenals. It is generally agreed that lack of the adrenotrophic hormone brought about by hypophysectomy leads to atrophy of the adrenal cortex, which can be prevented or reversed by injections of this hormone. However, whether the hormone regulates the function of the whole cortex, or only of some part of it, has not yet been determined. Another difficulty arises from the problem as to whether the adrenotrophic hormone is indeed a single substance or a complex.

It is, however, generally agreed that the pure adrenotrophic hormone must be free of the other pituitary hormones, and hence free of gonadotrophic and growth effects. These problems will not be dealt with here. We are concerned with another question which arose when we observed, after several months of treatment with adrenotrophic hormone, undertaken for an investigation which will be described in another paper,

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\*) Hans Hase died in February, 1948.

*that male and female immature rats responded quite differently to the same adrenotrophic hormone.* The female rats developed normally in accordance with the known properties of the hormone, while the male rats, compared with untreated rats of the same age, showed distinct signs of inhibition of growth and sexual development. This observation led us to wonder whether the different reactions to the hormone in the different sexes, so far not described in the literature, might not prove to be the expression of an antagonism between the male sex hormone and the adrenotrophic hormone. To study this question we had to devise an experiment in which this antagonism, if it exists, could be demonstrated more clearly and directly. The well known recovery test with rats, generally used for the testing of male hormones, seemed to be especially appropriate, in view of the reliability of the response to androgens. If the adrenotrophic hormone, administered at the same time as testosterone could suppress, or at least decrease the regeneration of the accessory sex organs in the castrated rat, then it would be obvious that this assumption would be proved correct. As for the relation between the adrenotrophic hormone and the oestrogens, we might have been satisfied with our observations made in the preliminary tests which agreed with the general view that they do not antagonise each other. However, in view of the importance we ascribed to this problem, we felt it necessary to administer the adrenotrophic hormone together with an estrogen in a similar manner, and to examine the effects produced. Here we made use of the well known property of oestrogens to inhibit the development of immature male rats. We thought that, provided there was an antagonism, administration of adrenotrophic hormone together with oestrogen would interfere with the oestrogenic inhibition. In the absence of antagonism, no interference with the oestrogenic effect would be seen. A third possibility that could not be excluded was that the two hormones would have a synergistic effect as indicated by certain findings in the preliminary experiments. We took the average weight increase up to eight weeks as a measure of sexual and body development. That

growth is not only dependent on the secretion of the pituitary growth hormone but is also influenced by the sex hormones was demonstrated by *Wilkins & Fleischman* (1946) who considerably increased growth in children by giving injections of testosterone.

## EXPERIMENTS

*Table I a.*

Weight increase in male rats treated with 1 ml. adrenotrophic hormone (ACTH)\*).

	4 weeks	6 weeks	8 weeks	No.
Treated animals	38 gm.	44 gm.	57 gm.	5 rats
Control animals (without ACTH)	37 gm.	59 gm.	72 gm.	5 rats

Table I a shows the result in two groups of 5 male rats, all aged about four weeks. The first group received 1 ml. of the adrenotrophic hormone 3 times a week; the other, the control group, was left untreated. The animals were weighed every fortnight, and the effect calculated as the average increase in each group of 5 rats; these figures are shown in the table. As may be seen, the treated rats, during four weeks, showed no difference in development as compared with the untreated rats. After six weeks, however, the difference became evident. After eight weeks, the control animals showed normally developed testes, while all the treated rats had very small testes. The weight of the testes in the control group was 2.4 gm., in the treated group 0.4 gm. The histological section of the testes of an untreated rat showed normal spermatogenesis up to mature spermatozoa. In the histological section of the treated rat, however, there was no complete

\*) ACTH prepared according to *James B. Collip*.



spermatogenesis, but only development and mitosis up to the formation of spermatocytes.

*Table I b.*

Weight increase in female rats treated with 1 ml. adrenotrophic hormone (ACTH).

	4 weeks	6 weeks	8 weeks	No.
Treated animals	33 gm.	54 gm.	61 gm.	5 rats
Control animals (without ACTH)	33 gm.	48 gm.	60 gm.	5 rats

Table I b shows the same experiment with immature female rats. Here there is no difference at all between the treated and untreated animals, either in sexual or body development.

*Table II a.*

Castrated male rats, three weeks after castration, treated for ten days.

	starting weight (gm.)	final weight (gm.)	seminal vesicle (mg.)	prostate (mg.)	adrenals (mg.)
(1). 0.1 ml	130	170	220	294	20
testosterone	155	185	330	352	22
propionate daily	175	190	302	306	20
(2). 0.1 ml					
testosterone	130	130	88	146	38
propionate daily	155	165	180	238	36
plus 1 ml. ACTH	175	175	146	185	52

Table II a gives the results of the experiments in which rats, three weeks after castration, were treated in the one group with testosterone propionate alone, and in the other group with testosterone propionate and the adrenotrophic hormone. The table shows the body weights of each of the three rats when the injections started, and the final body weights after ten days treatment. In addition it gives the weight of the seminal vesicle, the prostate and the adrenals.

Table II b.  
Average figures from Table II a.

	weight increase (gm.)	weight of seminal vesicle (mg.)	weight of prostate (mg.)	weight of adrenals (mg.)
(1)	28	284	317	21
(2)	3	137	189	42

The figures make it clear that the two hormones are antagonistic, as shown by the effect on the weights of the body, the secondary sex organs and the adrenals.

Table III.  
Comparison of the adrenotrophic hormone with Synoestron\*)  
in male rats.

weight increase in	4 weeks	6 weeks	8 weeks	No.
Control animals	36 gm.	64 gm.	78 gm.	5 rats
Synoestron*), 125 I. U. = 0.0125 mg.	30 gm.	50 gm.	56 gm.	5 rats
1 ml. ACTH + Synoestron (125 I. U.)	27 gm.	39 gm.	50 gm.	5 rats
2 ml. ACTH + Synoestron (125 I. U.)	22 gm.	34 gm.	44 gm.	5 rats

In this experiment we divided 20 male rats, about four weeks old, into four groups of five rats. The first group was untreated (control), the second was treated with a definite dose of a preparation of stilboestrol dipropionate, called Synoestron, the third group received the same amount of Synoestron together with 1 ml. of adrenotrophic hormone; and the fourth group was treated with the same dose of Synoestron and twice as much adrenotrophic hormone as in the third group. All the figures in the table are averages, each

\*) Synoestron (Teva brand of Stilboestrol dipropionate).

computed from five rats at the fourth, sixth and the eighth week of treatment. The figures show an inhibition which is increased by administering larger amounts of adrenotrophic hormone. At the end of the 8 weeks all the treated rats had very small testes while the control rats had testes of normal size. No autopsies were made as the experiment was continued for other purposes.

## DISCUSSION

All these experiments give evidence, either directly or indirectly, that there is — in more ways than one — a distinct antagonism between the male sex hormone and the adrenotrophic hormone of the hypophysis, while the female hormone (oestrogen) is not antagonised and possibly somewhat synergistic with this hypophyseal hormone. The question arises as to whether there is any reference in the literature on this observation. The limited literature at our disposal makes no direct reference to this sex difference in the behaviour of the adrenotrophic hormone. But many experimental findings, hitherto not considered from this point of view not only support our observation but their true significance now becomes clear. We refer particularly to all experiments performed to clarify the relation between the adrenals and the gonads, and which were usually performed on adrenalectomized or gonadectomized animals. Without the findings being regarded as very important, it was found by many investigators that removal either of the adrenals or of the gonads produced an action on the remaining organ which depended on the sex. According to *Hatai* (1913) gonadectomy of male rats results in an enlargement of the adrenals while gonadectomy of female rats is followed by adrenal atrophy, though no explanation was given for this sex difference. In trying to explain these findings, we shall first study the enlargement of the adrenal cortex. If we bear in mind that the size of the cortex is regulated by the adrenotrophic hormone of the pituitary, then we must come to the conclusion that castration augments the output of this hormone. This is not very surprising, since the stimulating

effect of castration on the pituitary function is well known. However, this at once raises the question as to why spaying of the females rat, followed also by pituitary hyperfunction, does not result in the same adrenal enlargement, but on the contrary is accompanied by adrenal atrophy. The answer to this question will be given later. It will lead to a very important problem in human pathology.

We turn now to the effects of adrenalectomy on the gonads of both sexes. *Jaffe & Marine* (1923) found that adrenalectomy in the rabbit was followed by atrophy of the testes and enlargement of the ovaries. *Freed, Brownfield & Evans* (1931) confirmed these findings in rats. Considering first the atrophy of the testes, the usual explanation ascribes this to lack of the adrenal androgens, which follows adrenalectomy. But this assumption is not very compatible with the slight effect which the adrenal androgens are believed to exert. In our opinion a better explanation is to be found if we take into consideration the more important relationship between the adrenals and the pituitary. In the same way as gonadectomy causes a pituitary hyperfunction, adrenalectomy produces a hyperfunctional state of the pituitary, mainly manifested by hypersecretion of the adrenotrophic hormone. This was very impressively demonstrated by *Houssay & Pinto* (1944). They found that the union in parabiosis of a normal rat, to another rat which had previously been deprived of the adrenal glands, resulted in the production of adrenal hypertrophy of the normal rat. From this they concluded that removal of the adrenal glands caused an increased production of the adrenotrophic hormone of the pituitary. This experiment had yet another interesting result. When *Houssay & Pinto* (1944) castrated the parabiotic rat before it was adrenalectomized, the hypertrophic effect on the adrenals of the other rat was still more marked. This observation supports our view expressed above that castration in male rats leads to the stimulation of the adrenotrophic hormone.

Returning now to the original question as to how the testes are effected by adrenalectomy, we take into consideration the

conclusion from our experiments, that an increased quantity of adrenotrophic hormone antagonises the action of the male hormone, and thus causes atrophy of the testes. This antagonistic action will be facilitated by the lack of the adrenal androgens; another question not yet answered by our own experiments arises in this connection, namely whether this assumed antagonism acts reciprocally. In that case, we should expect that an overdose of male hormone would suppress the adrenotrophic hormone. According to the way we interpret our experiments, this would mean that a large dose of testosterone should produce atrophy of the adrenal cortex. *Korenchevsky & Dennison* (1935) actually performed such experiments with rats and found adrenal atrophy. But they interpreted their finding as resulting from reciprocal inhibition between the gonadotrophin and the gonadal hormones, which comes into operation when either of the sex hormones predominates. Thus they interpreted the gonadotrophic inhibition as part of the general pituitary inhibition. But this explanation does not apply to the female rat in which according to the same authors, a large dose of oestrogen inhibits the gonadotrophins and at the same time brings about enlargement of the adrenal cortex. That this adrenal effect was not directly exerted by the oestrogens, as originally supposed, but mediated by the hypophysis was proved by *Selye & Collip* (1936) with hypophysectomized rats in which oestrogens caused no adrenal enlargement.

Now we come to the effect of adrenalectomy on the ovaries. These do not atrophy like the testes, but become enlarged, as demonstrated by many investigators, e. g. *Jaffe & Marine* (1923). We have already mentioned that removal of the adrenals leads to pituitary hyperfunction with increased output of the adrenotrophic hormone. As demonstrated by our experiments I b and III, there is no antagonism between oestrogens and this hypophyseal hormone. Therefore no reduction in size of the ovaries could be expected. The enlargement of the female gonads must be attributed to the fact that the missing adrenal androgens can no longer exert their inhibitory action

on the ovaries. This brings us, finally, to the solution of the question as to why ovariectomy of rats, though followed by pituitary hyperfunction, does not cause enlargement of the adrenals, in contradistinction to the observation on the male rat. It will be remembered once more that the mutual antagonism between the adrenotrophic hormone and the male hormone acts stimulating on the hypophysis after gonadectomy. No antagonism exists between the female hormone (oestrogen) and the adrenotrophic hormone. No stimulation is, therefore, exerted after ovariectomy. That is why no enlargement of the adrenals can take place in female rats after spaying. But as already pointed out, *Hatai* (1913) and *Masui & Tamura* (1927) have reported that the adrenals may even be smaller than normal after gonadectomy. This we take as an indication that in the female rat the whole hypophyseal response to gonadectomy occurs exclusively in the gonadotrophic activity at the expense of the adrenotrophic activity, which is thereby decreased. This shift-over is a well known phenomenon in multihormonal endocrine glands. But even if this adrenal decrease proved not to be a constant effect of spaying, it might still be concluded that gonadectomy causes two different hyperfunctional states in the pituitary: the one in the female in which the hyperfunction remains limited to the pituitary itself, and the other in the male in which the stimulus is also exerted on the adrenals.

It has already been indicated that we attach great importance to this mechanism in human pathology, and now we shall try to demonstrate its real significance. Provided that the above described correlation between gonads, pituitary and adrenals can also be accepted for the endocrine glands of man, a usual and legitimate transfer in endocrinology, it will prove useful to apply this knowledge to changes which occur in old age, and to examine what this involves in men and women, respectively. The decline in sex hormone production in men which accompanies senile involution of the testes, stimulates the secretion of the adrenotrophic hormone. Its increased output, on the one hand, leads to a further suppression of the

androgens, and on the other hand to an increased function of the adrenals. Which of the different hormone groups of the adrenals are thus affected we do not know with certainty, but it may be decisive for the health of the individual. In general we distinguish between three main hormonal groups of the adrenals, i. e., the salt-active, the sugar-active and the group of androgens. According to the investigations of *Selye* (1936), the hormones of the salt-active group secreted or supplied in excessive amounts display toxic properties leading to vascular diseases and hypertension. We intend to deal with this problem in a special paper. Here we are only concerned with the fact that a prolonged stimulation of the hypophysis is transferred to the adrenals and that this in all probability gradually leads to the secretion of large quantities of a hormone which is capable of producing vascular disease. At the same time it must be remembered that the stimulus resulting from the involution of the female gonads produces a different response in the hypophysis, which is not transferred to the adrenals. Therefore we cannot expect to find that the senile involution process in women tends to lead to vascular diseases. Indeed this is what is strikingly found in human pathology. In a recent paper *Cassidy* (1946) pointed out that men suffer three and a half times more than women from coronary diseases, and 70 per cent of them have hypertension. *Cassidy* quotes a report of the Mayo clinic in which the ratio is given as 4.3 to 1. Up to now no plausible explanation could be found for the higher morbidity in men. But our claim that the specific male reaction of the pituitary gland is the underlying cause for the prevalence of vascular diseases in men seems to be at variance with the fact that, although this mechanism usually works with age, not all old men suffer from vascular diseases. We have already noted that it has not been ascertained whether all or only a single group of hormones of the cortex are stimulated by the adrenotrophic hormone. Therefore it might be possible that, subject to certain conditions, the other non-toxic corticoids are more stimulated than the toxic group. For instance, the stimulation of the androgens would, by its anta-

gonism of the adrenotrophic hormone, involve a decreasing effect on its secretion, thereby preventing the fatal course to the toxic group and establishing a new balance between the adrenals and the pituitary gland. The favourable treatment of angina pectoris with testosterone, described by *Lesser* (1946), can be better understood with this interpretation than with the alleged property of testosterone to dilate the coronary vessels. However, the best therapy would be to break in time the vicious circle created by the antagonism and its influence on the salt-active group.

We can now point to some findings connected with age involution which provide some evidence of a different pituitary reaction in men and women. *Oesterreicher* (1932) found increased amounts of gonadotrophins in the urine of old women but not in the urine of men. *Hamilton, Catchpole & Hawke* (1945) comparing the urine titers of gonadotrophins in old and young men found no difference. In connecting this peculiar pituitary mechanism with the age involution of the gonads, we do not intend to assert that it comes into operation only at senility. On the contrary, we are of the opinion that the property of the pituitary gland to react differently in men and women appears even under other influences, for instances stress or emotion, provided they act for long periods and are mediated by the pituitary gland.

### SUMMARY

(1). It has been demonstrated by experiments with rats that an antagonism exists between the adrenotrophic hormone of the pituitary gland and testosterone, though there is no such antagonism between the adrenotrophic hormone and oestrogen.

(2). Experimental findings of other authors with regard to the relation between adrenals and the gonads in the male rat are quoted and interpreted with the help of this antagonistic relation.

(3). The different relation between the adrenals and the gonads in the female rat is explained by the fact that there is



no antagonism between the adrenotrophic hormone and oestrogen.

(4). The difference in the interrelation between the adrenotrophic hormone and androgens and oestrogens accounts for the sexual difference in the reaction of the pituitary gland to involution of the male and female gonads. Only in the male is the pituitary stimulation, which follows senile involution, transferred to the adrenals.

(5). The sex difference in hypophyseal reaction is regarded as a basis for the explanation of the phenomenon that the morbidity of vascular diseases like coronary insufficiency and hypertension are many times higher in men than in women.

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## THE EFFECTS OF ADRENOCORTICOTROPHIC HORMONE (ACTH) IN A CASE OF CHRONIC RHEUMATOID ARTHRITIS

BY

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The etiology of chronic rheumatoid arthritis is still obscure. The disease is far more frequent in women than in men; it is found at all ages, but most frequently in the fourth decade. A hereditary disposition is often found in this disease. Many features in the course of rheumatoid arthritis resemble those of chronic infections: the poor condition of the patient, the raised body temperature, the increased sedimentation rate, the anemia and achlorhydria. The pathological processes in the joints have an »infective« character. It is generally assumed that chilly and humid surroundings favour the outbreak of the disease.

The rheumatoid arthritis has a chronic progressive course, though it seems as if it possesses potential reversibility. Several remedies have been found to induce remissions, especially gold salts. This may also happen following febrile reactions to foreign protein. Transient amelioration has been observed during starvation, after surgical operations and, above all, during the course of hepatitis with jaundice and in pregnancy. The remission frequently starts in the 4th to 6th week

and disappears at about one month after parturition, independently of the duration of lactation. These observations do not fit in with the microbial theory but suggest the existence of some basic biochemical or hormonal disturbances (for further discussion and references, see: *Hench, 1949*).

For several years *Hench* and his collaborators have studied the effect of adrenal cortical extracts on the course of rheumatoid arthritis, and quite recently *Hench, Kendall, Slocumb & Polly* (1949) have published their astonishing results with *Kendall's Compound E* (= »Cortisone«) in 16 patients suffering from rheumatoid arthritis. Daily injections of 100 mg. Cortisone, or the acetate of this compound, brought about a marked clinical improvement in all the patients: the muscular and articular stiffness diminished, the articular tenderness and pain on motion were ameliorated. The general state improved, the appetite increased, and several patients experienced a marked sense of well-being. As a rule the symptoms began to recur within two to four days after discontinuation of the treatment. Two of the patients with a severe rheumatoid arthritis were further treated with 100 mg. doses of pituitary adrenocorticotrophic hormone (ACTH). The results of this administration were essentially identical with those resulting from the treatment with Compound E.

These remarkable observations were soon confirmed by other investigators (*Robinson et al., 1949; Thorn et al., 1949; and Wolfson et al., 1949*).

Reports of the results of biochemical and other analyses carried out during treatment of the patients with compound E are rather scanty. The sedimentation rate decreases, the globulin content of the plasma diminishes. The uric acid excretion increases, and there is a fall in the number of circulating eosinophil leucocytes. ACTH sometimes brings about hypertension, glycosuria, and in one patient acne, hirsutism and amenorrhea occurred and the general appearance of the patient was reminiscent of Cushing's syndrome.

The effects of the various adrenal cortical steroids in the normal human organism have not been described to any great

extent. Recently, the metabolic and biochemical changes obtained by ACTH administration to normal subjects have been thoroughly investigated, e. g. by *Mason et al.* (1948), *Forsham et al.* (1948), and *Sayers et al.* (1949). The most important findings described in these publications are discussed below.

## OWN INVESTIGATIONS

As soon as the reports of the effects of Kendall's Compound E and of ACTH in cases of chronic rheumatoid arthritis had reached Denmark, we decided to try the treatment in suitable cases. The aim of the investigations was to examine the clinical effects of these remedies and to perform as many biochemical, hematological and hormonal analyses as possible. It proved to be impossible to obtain the preparations from U. S. A., but through the courtesy of Dr. *Frederik Paulsen, Nordiska Organon, Stockholm*, we were given an amount of Cortrophin (ACTH), sufficient to carry out the first of the planned experiments.

### *Material and methods.*

The ACTH preparation used was of porcine origin. With the permission of the makers some of their assay results are reported below. The activity of the preparation was determined by Sayer's method (see: *Sayers, Sayers & Woodbury*, 1949) and gave the results summarized in Table 1.

Table 1.

The potency of the Cortrophin preparation used in the present investigation. By the courtesy of Organon.

Dose in $\gamma$ per 100 gm. body weight	Mean ascorbic acid decrease, in $\gamma$ per 100 mg. adrenal*)
0.33	46
1.0	76
3.0	125
9.0	143

\*) The figures in this column represent the average result from 20 hypophysectomized rats.

In human subjects a 50 to 80 per cent decrease in the number of eosinophil leucocytes occurred after a single injection of 25 mg. of the preparation. It possessed a slight *pressor activity* (0.02—0.04 I. U. per mg.) and a slight oxytotic activity (0.02 I. U. per mg.) Prolactin was not demonstrable.

*Blood sugar* was determined by the method of Hagedorn & Norman Jensen (1923); *total plasma proteins*, by the technique of Henriques & Klausen (1932). The *electrophoretic examinations of the plasma proteins* were carried out in the State Serum Institute with the Tiselius apparatus by the biochemist Mr. Birch Andersen.

*Uric acid* was determined by the uricase method of Prætorius (1949); *cholesterol in serum* by a modification of Brun's technique (1935). For the determination of *sodium in serum* the method of Bierring & Nielsen (1948) was used; and *plasma bilirubin* according to With (1943).

*Urinary 17-ketosteroids* were assayed by Hamburger's micro-method (Hamburger & Rasch, 1948). The *glucocorticoids* in the urine were measured by the glycogen deposition test; the procedure of Venning, Kazmin & Bell (1946) was strictly followed. The standard curve given by these investigators was used for the calculation of the »glycogenic units«, as the lack of Kendall's Compound E made it impossible for us to establish our own calibration curve. This procedure seems justifiable, as the values found in our case before and after ACTH administration agree with the normal values of Venning *et al.* In any case, the *relative* values for the excretion from day to day must be fairly accurate. The *urinary reducing corticoids* were measured by the method of Heard & Sobel (1946) and Heard, Sobel & Venning (1946), modified by Sprechler (1949).

#### *Case record.*

M. L.-R. J. No. 968/49 53 years old married woman. 3 pregnancies, no abortions. 1913 appendectomy. 1921 tonsillitis and otitis media complicated with mastoiditis. 1923 operation for

uterine prolapse. 1924 otitis media. Since 1935 the patient had suffered from a progressive chronic rheumatoid arthritis and was repeatedly admitted to hospitals. She was treated with gold salt four times, last treatment in 1947.

Admitted at Rigshospitalet, Med. Dept. A, on Jan. 31, 1949. The following three months she was treated with physical therapy but with only little success. Before the treatment with ACTH the patient showed a typical rheumatoid arthritis in advanced stage. There were pain, stiffness, and capsular swelling of the following joints: shoulders, elbows, hands, metacarpal, interphalangeal, knees, ankles, and metatarsophalangeal. The right fingertips failed to reach the palm by 1 cm. in maximal flexion and those of the left hand 5 cm. She was unable to move a spoon to her mouth with the right hand, could not brush her hair, and walked with great difficulty. Furthermore there was an infiltration of fibrous tissue as large as the palm in the left trochanteric region; even the most energetic physical treatment had been unable to lessen or remove this.

From June 25th she received daily intramuscular injections of ACTH, the doses being gradually increased from 25 mg. to 100 mg. After two injections of 100 mg. the dose was diminished to 75 mg. which was given for 5 days; she then received 50 mg. daily for 4 days and finally one injection of 25 mg. Altogether she received 950 mg. ACTH in the course of 15 days. The preparation was given as a single injection in the morning, with one exception, the last 100 mg. dose being divided into two. Later on the patient was treated with daily injections (i. m.) of testosterone propionate in oily solution. When not injected with these remedies, the patient received daily placebo injections of saline.

### *Clinical findings.*

As early as one day after the beginning of the treatment a remarkable subjective improvement was noticed. The articular and muscular functions improved, and in the course of the following days the swelling of the joints decreased. Hy-

drops developed in the knees followed by well marked streptus localized to the capsule tissue; also the tissue of other joint capsules became smoother and less tender. The circumference of the right ankle-joint was decreased by 2.5 cm., and the strap of her wrist-watch had to be considerably tightened. In the course of a few days she was able to give a firm grasp of the hand and to clench her fist. Shortly afterwards she was able to climb stairs. After the twelfth injection she was able to produce a work performance of 400 kgm./min. for some minutes on bicycle-ergometer. The infiltration of the left thigh disappeared completely, and the 36-year-old appendectomy scar became tender and narrower. Two days after the last injection, the symptoms recurred. The articular pains, tenderness, stiffness and swelling reappeared and in the course of one week the state of the patient was as before the treatment, furthermore the fibrous infiltration gradually returned. Slight feeling of pressure in the chest and hot flushes were the only untoward reactions observed. The blood pressure, pulse rate, body temperature and body weight did not change significantly. The subsequent treatment with testosterone propionate had no effect whatever on her symptoms.

*Laboratory findings* (see Figs. 1 to 3).

The *urinary output* averaged 750 ml./24 hours, with the usual day-to-day variations. The *fasting blood-sugar* was slightly raised from the day after the first injection and remained somewhat unsteady up to one week after the last injection. The maximal fasting value was 158 mg. per cent. *Glycosuria* appeared for some days, the maximal excretion of sugar being 5 gm./24 hours. *Ketonuria* did not occur.

The *total plasma proteins* decreased during the treatment from about 9 to 7 gm. per cent. The albumin fraction was unchanged; the globulin fraction decreased from about 4 to 2.3 gm. per cent. By electrophoretic examinations the decrease was found to be due to a diminution of the  $\gamma$ -globulins. The decrease persisted for some days after discontinuation of the treatment. Testosterone propionate had no effect on the plasma proteins.

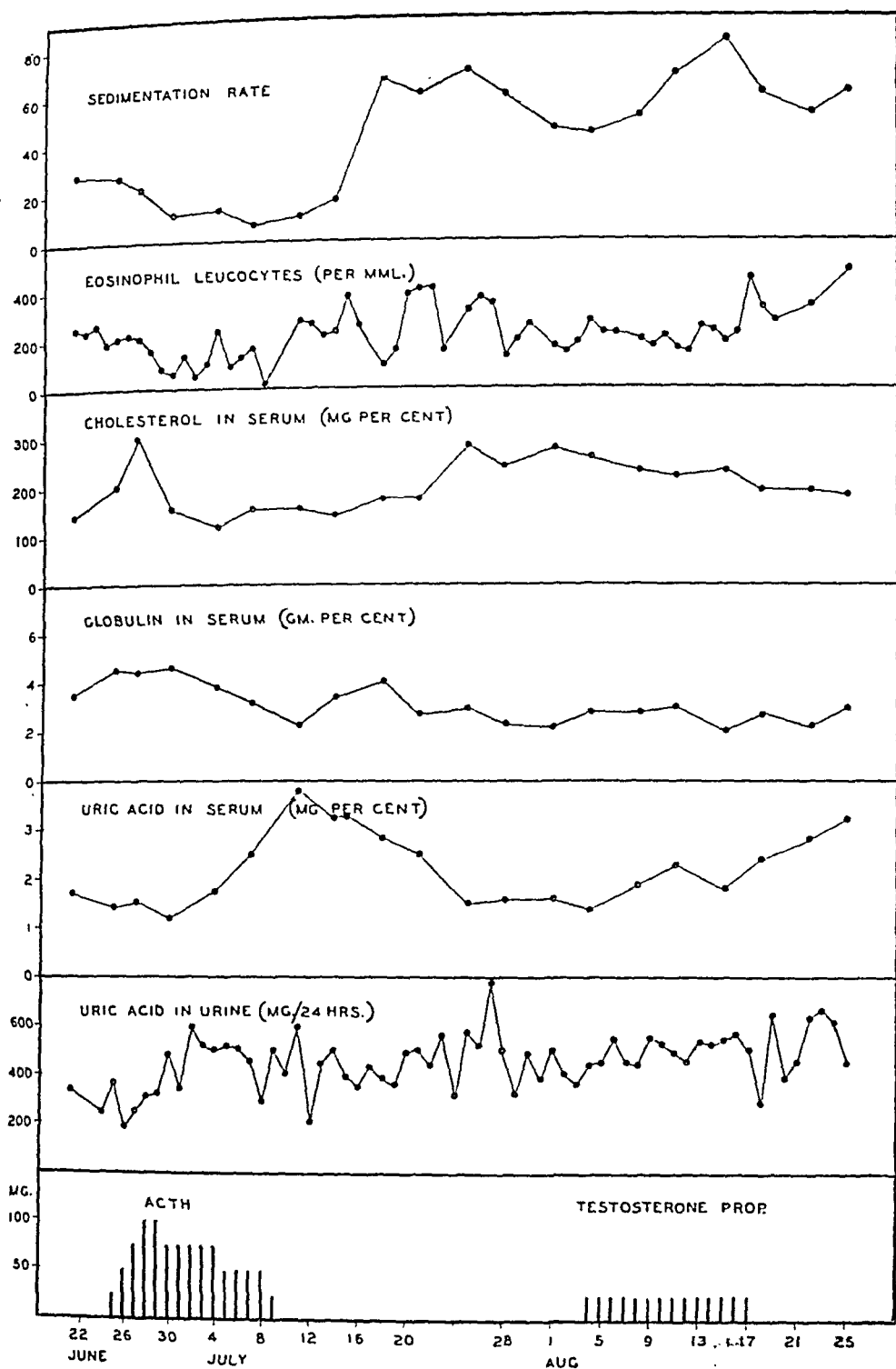


Fig. 1.  
Hematologic and metabolic changes in the present case.



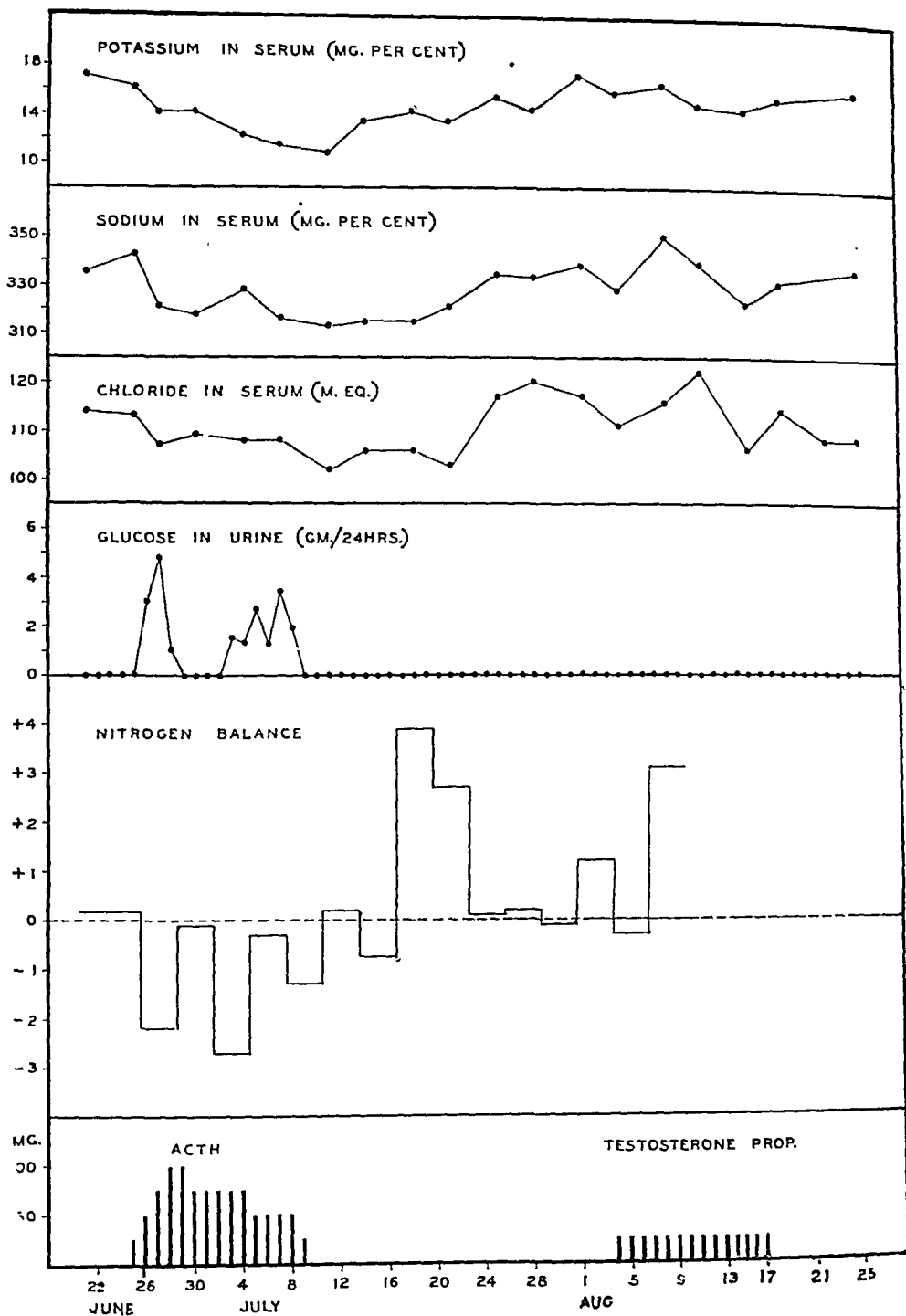


Fig. 2.

Metabolic changes in the present case.

*Nitrogen balance.* The patient was put on a constant diet, the protein content of which was determined. The excretion of nitrogen in the urine and stools was measured. During treatment there was a negative nitrogen balance, followed by a post-treatment period of positive balance.

*Uric acid in serum* increased from about 1.5 to 3.8 mg. per cent during the ACTH administration and for a few days afterwards; it then gradually decreased. During and after the treatment with testosterone propionate the values were slightly increased. The *uric acid excretion* was somewhat difficult to evaluate, as the pre-treatment observation period was rather short; a moderate increased excretion occurred during the treatment. The patient was not put on a purine-free diet.

The content of *cholesterol in serum* showed an initial increase, followed by a decrease to normal values. A gradual increase subsequently occurred, beginning during the last days of the injection period and reaching a plateau about two weeks after the last injection.

The *basal metabolic rate* was rather constant at 90 per cent, probably exhibiting a slightly falling tendency during and shortly after the treatment.

*Potassium, sodium, and chloride.* A marked fall in the potassium content of the serum occurred during the treatment with ACTH from 17.3 to 10.9 mg. per cent. The sodium content likewise decreased, whereas the decrease in chloride was insignificant. No changes in the electrolytes were observed during the testosterone propionate treatment.

*Hematologic examinations.* The *blood sedimentation rate* decreased from 28 to 7 mm. during the administration of ACTH, but afterwards rose to 60 mm. There were no significant changes in the *hemoglobin content* or in the *erythrocyte content*. The hgb. was about 80 per cent but when the patient got worse after discontinuation of the treatment, the hemoglobin gradually fell to 50 per cent and then slowly increased. The *blood platelet* count was constant. The number of circulating polynuclear leucocytes varied apparently independently of the injection period; the *eosinophil* leucocytes decreased a

little, but no significant changes in the number of the lymphocytes were noticed.

The *bilirubin content of plasma* was rather constant (0.20 to 0.30 mg. per cent). A transient increase (up to 0.58 mg. per cent) occurred following treatment.

*Serologic examinations.* The *anti-streptolysin titer* remained low during the whole observation period. The *streptococcal agglutination titer* decreased from 1:640 to 1:160 during the ACTH administration, but marked variations had occurred during the preceding months.

The *electrocardiogram*. Slight changes were seen in the terminal complexes. The duration of systole was prolonged a little, but not to a pathological degree.

The *17-ketosteroid excretion* was low in the periods when no injections were being given. The average value (2.7 mg./24 hours) was half the normal average for a woman of her age and at the lower limit of the normal range (*Hamburger*, 1948). Immediately after the beginning of the injections the excretion increased simultaneously with the increasing doses of ACTH. The increase continued for a couple of days after a diminution of the daily dose to 75 mg.; a first maximum (16.5 mg.) occurred on the 8th day of the treatment, but then the excretion decreased to 9.1 mg. The treatment was continued with smaller doses (50 and 25 mg. per day) and another steep rise occurred, the highest value (17.9 mg./hours) being observed on the first day after the last injection. In the course of the next 24 hours the 17-ketosteroid excretion had fallen to almost pre-treatment level. During the treatment with testosterone propionate (25 mg. daily for 14 days) the excretion increased and reached a maximum (9.2 mg.) on the 7th day, then gradually declined.

Separation of  $\alpha$ - and  $\beta$ -17-ketosteroids by the digitonin precipitation method of *Frame* (1944) was performed on several of the urines. The average percentage of  $\beta$ -fractions was 4.4 during ACTH administration; in the period without any treatment the figure was 1.7; during the testosterone propionate administration the  $\beta$ -fractions averaged 1.2 per cent.

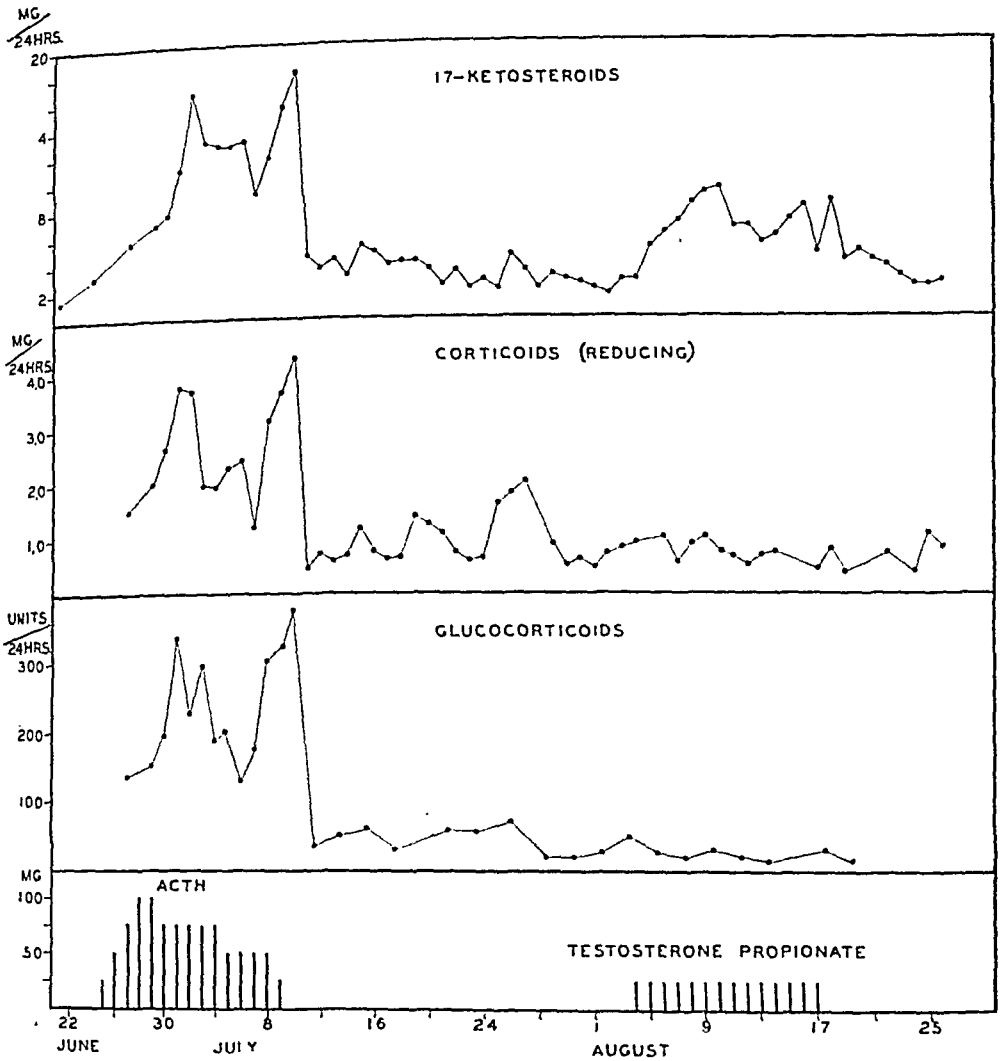


Fig. 3.

Urinary excretion of steroids in the present case.

Mason *et al.* (1948) found no digitonin precipitable fraction in their extracts.

The urinary excretion of reducing corticoids was examined from the third day of the treatment period. The excretion curve showed a remarkable resemblance to that of the 17-ketosteroids, i. e. two peaks (at 3.76 and 4.25 mg./24 hours, respectively) and a period of low excretion between them. The second maximum occurred on the first day after discontinua-

tion of the ACTH administration but the next 24-hour urine contained only 0.44 mg./24 hours. The excretion was somewhat irregular during the next two weeks. Treatment with testosterone propionate was not accompanied by any increase in the content of reducing corticoids.

The glucocorticoid excretion agreed so closely with the values obtained chemically that a description of the excretion curve would be a mere repetition. In the course of 24 hours the excretion fell from the second maximum (375 glyc. units) to above one tenth of this value. The daily variations in the post-treatment excretion were less marked than those observed with the chemical method.

## DISCUSSION

The clinical improvement of the patient during the administration of ACTH was very remarkable. In no respect did it differ from the results reported by *Hench et al.* (1949), the symptoms promptly recurring when the treatment was discontinued.

The changes observed in the composition of the blood and urine during the ACTH administration were, in most instances, of the same nature as those reported by *Mason et al.* (1948), *Forsham et al.* (1948) and *Sayers et al.* (1949) viz., a decrease in the sedimentation rate and in the number of eosinophil leucocytes, negative nitrogen balance, hyperglycemia, glycosuria, a decrease in the amount of potassium in the serum, and an increase in the uric acid concentration. Further comparative data are given in Table 2. The excretion of 17-ketosteroids, reducing corticoids and glucocorticoids increased considerably, the maximal values being 6—7 times the average control value for the 17-ketosteroids and the reducing corticoids, while the maximal excretion of the glucocorticoids exceeded the average control value by 15 times.

These observations seem to indicate that a stimulation of all the multiple functions of the adrenal cortex had taken place. The effects of the ACTH treatment were confined to

the period of treatment and to the first two days after discontinuation of the injections. In this respect no difference seems to exist between the »stimulation therapy« with the hypophyseal »-trophic« hormone and the »substitution the-

Table 2.

Biochemical and hematologic effects in man during administration of adrenocorticotrophin. Comparison of data from the literature with those of the present investigation.

Laboratory examinations	Mason et al (1948) <sup>1)</sup>	Forsham et al. (1948) <sup>2)</sup>	Sayers et al. (1949) <sup>3)</sup>	Present case <sup>4)</sup>
Urinary 17-ks. . . . .	+	+	+	+
Urinary glucocorticoids . . .	+	+	+	+
Total urinary nitrogen . . . .	(+)	(—)	+	+
Uric acid in urine . . . . .		+	+	+
Uric acid in serum . . . . .	0	—	(+)	+
Blood sugar . . . . .			+	+
Glucose in urine . . . . .		0	+	+
Plasma cholesterol . . . . .	—			+ — — —
$\gamma$ -globulins . . . . .	0	0	0	—
CO <sub>2</sub> in plasma . . . . .	+	(+)	0	
Potassium in plasma . . . . .	0		—	—
Potassium in urine . . . . .	0	+	+	
Sodium in plasma . . . . .	0		0	—
Sodium in urine . . . . .	0	—	(—)	
Chloride in plasma . . . . .	0		0	(—)
Chloride in urine . . . . .	0	—	(—)	
Lymphocytes . . . . .		—	—	0
Eosinophil leucocytes . . . . .		—	—	—

1) Normal young woman, daily i. m. injections of 25—100 mg. ACTH.

2) Normal and pathol. cases. Single and repeated i. m. injections of ACTH.

3) Two normal men. Single intravenous infusion of 50 and 100 mg. ACTH.

4) 53 years old woman with rheumatoid arthritis. Repeated i. m. injections.

0 = no change; + = increase; — = decrease

(+) = slight increase; (—) = slight decrease.

rapye with the cortical hormone, a fact which emphasizes the special nature of the hypophyseal-adrenal relationship.

The well marked two-peak excretion curves for 17-ketosteroids and corticoids raises certain questions. The remarkable agreement of the three curves (see Fig. 3) rules out the possibility that the cause might be sought in technical variations involved in the analytical procedures. They must undoubtedly reflect important changes in the production and secretion of steroids by the adrenal cortex. A study of the mechanism of the hypophyseal-adrenal relationship, recently summarized by *Sayers & Sayers* (1948), seems to offer a reasonable explanation of the phenomenon.

ACTH acts as a catalyst in the series of reactions involved in the transformation of cholesterol to cortical hormones. A depletion of sudanophil substance, cholesterol and ascorbic acid from the cortex takes place (*Long*, 1947, and *Ducommun & Mach*, 1949). In the present case the first seven injections (25, 50, 75, 100, 100, 75 and 75 mg., resp.) accelerated the production of the cortical hormones at a rate far exceeding the restoration of precursors of the hormones. The secretory capacity of the cortex is consequently exhausted, and the production, release and excretion of the hormones decrease in spite of continued ACTH administration. It is not unlikely that a continuation of the high doses might have resulted in a complete and fatal break-down of the cortical functions. Before the excretion had reached the minimum, the daily dose of ACTH was, however, decreased to 50 mg. per day for 4 days and then to 25 mg. (the last injection). At this dose level the rate of new formation of cholesterol ester exceeds that of the production and release of cortical hormones, allowing the cortex to recover. It is reasonable to assume that the ACTH injections have produced a hypertrophy of the cortex, and that the hypertrophied cortex was able to respond to the ACTH stimulation; the second maximum in the steroid excretion thus reflects the increased production and secretion of cortical hormones.

During the treatment with *testosterone propionate* the uri-

nary 17-ketosteroid excretion increased. An additional excretion of about 70 mg. took place, and it could be calculated (according to *Hamburger & Kaae*, 1949) that about 24 per cent of the testosterone propionate injected had been excreted as 17-ketosteroids. The additional excretion of 17-ketosteroids during the ACTH administration amounted to about 118 mg. From these figures it can be calculated that the ACTH treatment has had the same effect upon the 17-ketosteroid excretion as the injection of the total dose of about 600 mg. of testosterone propionate.

During the administration of testosterone propionate the only significant effect observed was the increased 17-ketosteroid excretion. As all the other changes brought about by the ACTH administration were absent or insignificant, it may be concluded that the hematological and metabolic changes cannot be due to the 17-ketosteroids or their precursors, but must be produced by an increased elaboration of other adrenal cortical steroids.

### SUMMARY

A 53 years old woman suffering from severe chronic rheumatoid arthritis of 14 years' duration was treated with adrenocorticotrophic hormone (ACTH) for a period of 15 days. The total dose was 950 mg., the daily dose varying between 25 and 100 mg.

The clinical effects closely resembled those reported by other investigators. The articular swellings, pain and tenderness on motion rapidly diminished. The articular and muscular functions were remarkably improved. During the treatment she could get out of bed, walk without pain, and climb stairs unaided. Her appetite improved, and she experienced a feeling of well-being. A remarkable effect on the fibrous tissue was observed. The blood sedimentation rate decreased, the total plasma protein decreased, due to a diminution of the  $\gamma$ -globulins. There was a negative nitrogen balance and an increase in the uric acid concentration in the serum. Hyper-



glycemia and glycosuria occurred. The serum potassium and sodium decreased. The number of circulating eosinophil leucocytes decreased.

The urinary excretion of 17-ketosteroids, reducing corticoids and glucocorticoids increased 6 to 15 times. The excretion curves for these substances were remarkably alike and exhibited two maxima and an intervening depression. This peculiar excretion pattern was thought to be due to a temporary exhaustion of the cortical functions, caused by an overstimulation by the 100 mg. doses of ACTH.

A few days after discontinuation of the treatment the symptoms of the disease reappeared and the laboratory findings returned to pre-treatment conditions. The only effect of a subsequent treatment with 350 mg. testosterone propionate for 14 days was an increased output of 17-ketosteroids.

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## THE EFFECT OF DESOXYCORTICOSTERONE ACETATE (DCA) AND SODIUM CHLORIDE ON BLOOD PRESSURE AND RENAL FUNCTION\*)

BY

ROLF LUFT and BJÖRN SJÖGREN

The effect of DCA, with or without an extra supply of sodium chloride, on blood pressure and renal function has been studied by a number of authors in man and in animal experiments. *Selye* and his coworkers (1943—46), in a series of investigations, observed that the administration of large doses of DCA and sodium chloride in birds, rats and dogs gave rise to severe renal changes, resembling nephrosclerosis, and to a rise in blood pressure. According to *Friedman et al.* (1948) they were able to confirm these observations. At the same time they examined the renal function in their experimental animals (rats) and found, inter alia, a reduced renal blood flow simultaneously with a rise of blood pressure. To the present authors, however, these results seem to indicate that DCA does not cause any marked changes of blood pressure and renal function in intact animals. *Bechgaard & Bergstrand* (1949), by

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\*) The DCA used in the present investigation was »Percorten« which was kindly placed at our disposal by CIBA Produkter AB, Stockholm.

Aided by a grant from Karolinska Institutet, Stockholm.

giving DCA to their rats produced slight degeneration of the tubular cells of the kidney, but no changes in the vascular system or glomeruli, and no significant increase of blood pressure after four months' treatment. Nor could *Swingle et al.* (1941) bring about a rise in blood pressure in normal rats by means of DCA. *Grollman* and coworkers reported in 1940, that the administration of DCA for one week raised the systolic blood pressure in normal rats by 50—80 mm. These authors obtained a similar rise of blood pressure with testosterone, progesterone and stilboestrol. *Knowlton et al.* (1946, 1949) were unable to produce hypertension in normal rats with DCA and sodium chloride, but obtained a considerably increased blood pressure with DCA when treating the animals with a nephrotoxic serum. *Rodbard & Freed* (1942) studied the effect of DCA on healthy dogs and on dogs with Goldblatt-kidneys. Some of the animals in each group reacted with a marked increase of blood pressure. Similar results were obtained by *Davis et al.* (1948). *Kuhlmann et al.* (1939) obtained, by the administration of DCA to a normal dog, a rise in blood pressure of 20 mm Hg., in a Goldblatt-dog a rise of 40 mm Hg. *Summers* (1948), using DCA and NaCl, failed to obtain a blood pressure higher than that found in control dogs or dogs given NaCl alone.

Hypertension may occur in the treatment of Addison's disease with DCA and sodium chloride. This increase of blood pressure takes place gradually, reaching its maximum after 4—16 weeks of treatment (*Ferrebee et al.*, 1939, *Thorn et al.*, 1939—41, *Wilder*, 1940, *Mc Cullagh & Ryan*, 1940, *Soffer et al.*, 1940, *Engel et al.*, 1942, *de Gennes et al.*, 1947). A further increase of blood pressure in cases of essential hypertension by means of DCA and sodium chloride was described by *Perera & Blood* (1947) and *Perera* (1948). These authors, however, produced only a moderate rise of blood pressure and this only after a fairly long period of DCA and sodium chloride administration in healthy subjects (*Perera et al.*, 1944). *Raab* (1942) found no increase in blood pressure in healthy subjects with this treatment, but noticed that the subjects reacted,

during the DCA treatment, with a greater increase of blood pressure to the administration of adrenaline.

Summing up, it may be inferred that hypertension has not been produced in healthy subjects by the administration of DCA and sodium chloride. There is still a difference of opinion as to whether DCA can give rise to an increase of blood pressure in normal animals. It is possible that a disordered renal function is a prerequisite for such a hypertension. The effect of DCA on the renal function in man has only been examined in cases of Addison's disease (*Talbott et al.*, 1942, *Waterhouse & Keutmann*, 1948, *Luft & Sjögren*, 1949 c).

The present authors observed, after the administration of DCA and sodium chloride to healthy subjects, a rise of the systolic pressure of 10—25 mm Hg, and of the diastolic of 5—10 mm Hg (*Luft & Sjögren*, 1949 b). In one case of pan-hypoadenopituitarism, DCA and sodium chloride caused a rapid increase of blood pressure from 90/50 to 170/105 mm Hg, though only after premedication with thyroxin (*Luft & Sjögren*, 1949 a). In one case of Addison's disease, complicated by a chronic glomerulonephritis, a *rapid* increase of the blood pressure to 180/110 mm Hg was also noted (*Luft & Sjögren*, 1949 c).

In this paper, the effect of DCA and sodium chloride was studied on blood pressure and renal function in healthy subjects and in subjects with disturbed renal function.

## MATERIAL AND METHODS

The material comprises 24 subjects, i. e. 15 females and 9 males (Table 1).

*Cases 1—3.* Healthy subjects.

*Case 4.* Patient recovering from an acute infective hepatitis.

*Cases 5—7.* Healthy subjects with hypotension.

*Cases 8—9.* Had moderate nervous anorexia.

*Case 10.* Chronic renal disease. Autopsy revealed only moderate vascular changes. The kidneys and especially the cortical layers were considerably enlarged owing to a marked lipoidosis of the parenchyma.

*Case 11.* Chronic pulmonary disease with presumably renal amyloidosis.

*Case 12.* Addison's disease with chronic glomerulonephritis.

*Case 14.* Chronic glomerulonephritis.

*Case 14.* Nervous anorexia. Two years previously, attack of hematuria. Glomerulonephritis?

*Case 15.* Albuminuria for a short period after parturition a few years previously. Glomerulonephritis?

*Case 16.* Chronic glomerulonephritis.

*Case 17.* Nervous anorexia, pulmonary tuberculosis. Glomerulonephritis?

*Cases 18—19.* Postural hypotension.

*Case 20.* Subchronic hepatitis.

*Case 21.* Duodenal ulcer. Renal disease?

*Case 22.* Chronic glomerulonephritis.

*Case 23.* Cirrhosis of the liver.

*Case 24.* Addison's disease and chronic glomerulonephritis.

We do not propose to give a detailed account of these cases. Here, it will suffice to state that albuminuria, increased N. P. N. and pathological urinary sediments occurred in cases 10, 12, 13 and 16.

During the week prior to the test, the patients were submitted to a hospital diet deficient in salt with a calculated content of a maximum of 3 gm. of sodium chloride and a constant supply of fluid. Daily controls of the blood pressure, hematocrit readings and number of red blood cells, diuresis, chloride excretion and body weight were performed. Inulin and diodrast clearances as well as the total protein, albumin and globulin content in the serum were determined before and after medication. Apart from the routine tests, the examination of the patients also included water tolerance tests (1000 ml.).

The analyses of inulin and diodrast were done acc. to *Corcoran & Page* (1939), *Alving et al.* (1939) and *Alpert* (1941).

The diodrast was given by intramuscular injection acc. to *Josephson* (1945). Each clearance test was performed in three periods, each period being terminated by catheterization of the bladder and insufflation of air.

The blood pressure measurements were carried out each morning by the authors with a Baum Mercury Manometer before the patients had got out of bed. The hematocrit determinations were made acc. to *von Porat* (1948), the other examinations being performed according to routine procedures.

Peripheral vascular resistance in the kidneys was calculated acc. to *Lamport* (1943).

For evaluation of the clearance values, the results obtained in 10 subjects were used, i. e. in whom no signs of disturbed renal function or blood pressure were ascertainable. The results were, as follows:

a) *Glomerular filtration*. Mean value = 117.0 ml/min.  $\pm$  8.3. Standard deviation ( $\sigma$ ) = 26.3.

b) *Renal plasma flow*. Mean value = 572.6 ml/min.  $\pm$  24.3. Standard deviation ( $\sigma$ ) = 76.8.

c) *The error of the method* ( $\sigma_i$ ) of the inulin and diodrast clearances was calculated by means of analysis of variation (*Fisher*, 1936) in 60 cases (normal and abnormal) during 170 periods. Results: for inulin clearance 20.8 ml/min. for each period and 12.0 for each complete clearance of 3 periods, for diodrast clearance 50.3 and 29.0 ml/min., respectively.

## RESULTS

1. The results of these investigations have been compiled in Table 1, below:

We divided our cases into two groups, according to the rise of blood pressure after the administration of DCA. One group includes cases 1—14 in Table 1, the other cases 15—24. The cases in the former group showed, after having been given DCA and sodium chloride, a systolic blood pressure that was

lower than 150 mm Hg. In the latter group, the systolic pressure rose to over 150 mm Hg. In Group I, the increase of systolic pressure was less than 30 mm Hg, while in Group II it exceeded 40 mm Hg, except in case 23 where there was a rise of 37 mm Hg. The mean values of the initial pressures, calculated from the mean values of the blood pressure in each individual case for 5 days before the administration of the DCA, and the mean values of the final pressures, calculated from the mean values of the blood pressures in each individual case during the last 5 days of the DCA treatment and, finally, the mean values of the blood pressure increase in both groups are recorded in Table 2.

The mean blood pressure in Groups I and II, for each day during the last five days before the treatment, and five days before the end of the treatment, is shown in Fig. 1.

It is evident from Table 2 that the rise of blood pressure was statistically significant in both groups.

2. The hematocrit values fell with DCA administration, in Group I by  $6.7 \pm 0.8$ , in Group II by  $6.6 \pm 0.9$ . The number of red blood cells in Group I fell by  $0.7 \text{ mill/mm}^3 \pm 0.1$ , in Group II by  $0.6 \text{ mill/mm}^3 \pm 0.1$ . The body weight in Group I (cases 12—14 excluded) increased by  $3.0 \text{ kg.} \pm 0.3$ , in Group II by  $2.4 \text{ kg.} \pm 0.4$ . Thus, no definite differences existed between the groups as regards hemodilution and water retention.

3. In Group II the patients were selected depending on the degree of blood pressure increase. In all the cases in Group II the renal plasma flow was more than  $2 \sigma$  below the normal value, except for cases 20 and 22. However, case 22 showed a glomerulonephritis in the case history. In case 16 and 24, no diodrast clearance could be made, but the values of the glomerular filtration (9 and 44 ml. respectively) were the lowest in this group and, consequently, it may be assumed that the blood flow was reduced to a corresponding extent. The glomerular filtration was significantly decreased in Group II ( $P < 0.001$ ).

4. In twelve cases, the glomerular filtration and renal blood flow, were measured before and after a period of DCA and so-



Table 1  
Effect of DCA and Saline on Blood

Case	Sex	Age Years	Treatment DCA + NaCl					Albumin Globulin in Serum Per Cent		Hema- tocrit Reading		Red Blood Cell Count Mill./Mm <sup>3</sup>		Body Weight Kg
			DCA Mg./D.	NaCl G./D.	Days	Ede- ma	Thy- roxin Mg./D.	1.	2.	1.	2.	1.	2.	
1. M.R.	M.	25	20	10	17	0	—	—	—	56	47	5.2	4.1	64.5
2. F.A.	M.	30	20	10	14	0	—	—	—	58	50	5.5	4.5	75.2
3. K.G.E.	M.	37	20	10	14	0	—	—	—	50	44	5.0	4.5	74.0
4. F.O.K.	M.	37	20	10	14	0	—	—	—	—	—	—	—	—
5. I.G.M.W.	F.	34	20	10	10	0	—	—	—	42	35	4.2	3.5	—
6. E.L.B.	F.	31	20	10	24	0	—	5.0	—	51	37	4.5	3.2	51.8
								1.6						
7. E.L.	M.	35	20	10	18	0	—	5.0	—	45	39.5	—	—	60.9
								2.1						(35.0)
8. I.M.H.	F.	19	20	10	17	0	—	4.3	4.3	32	28	3.0	2.6	33.0
								2.2	1.2					
9. I.B.S.	F.	25	20	10	16	0	—	4.8	5.1	43	39	4.1	3.6	47.5
								1.8	1.5					
10. A.E.E.	F.	56	20	5	12	—	—	2.1	1.4	49	38.5	4.8	4.1	48.0
								2.4	2.6					
11. M.Z.	F.	52	20	5	15	0	—	3.7	3.4	40	34	4.0	3.1	39.1
								3.2	2.8					
12. K.I.E.	M.	54	20	5	8	+++	—	—	—	36	29	3.6	2.8	76.5
13. A.S.G.	F.	43	20	5	7	+++	—	3.6	—	22	21	2.9	2.9	34.5
								1.6						
14. R.I.E.	F.	36	20	10	16	+++	—	1.6	3.5	44	38	4.2	3.6	39.0
								2.1	2.3					
			20	10	6	---	3	3.3	3.4	37	28	3.8	3.0	36.5
								1.7	1.3					
15. L.N.	F.	36	20	5	14	+	—	4.6	1.8	45.5	36	3.9	3.3	54.0
								2.1	1.8					
16. V.D.L.	F.	56	20	3-5	14	0	—	4.1	4.2	—	—	—	—	68.8
								2.8	1.9					
17. G.M.E.	F.	33	20	10	16	0	—	3.3	3.3	42	37	4.0	3.2	31.8
								2.1	1.9					(45.0)
			20	10	22	0	3	3.5	4.1	38	33	3.5	2.9	41.3
								2.3	1.7					
18. K.O.V.E.	M.	48	20	10	59	0	—	—	—	—	—	—	—	62.0
19. I.S.M.	F.	53	20	10	26	0	—	—	—	44	37	4.1	3.2	63.5
20. K.V.L.	F.	56	20	10	15	0	—	4.9	4.6	41	36	3.8	3.5	—
								2.3	1.9					
21. G.V.T.	M.	63	20	10	17	0	—	—	—	55	46	5.2	4.5	67.0
22. K.N.	M.	55	20	10	13	0	—	—	—	47	44	4.5	4.1	62.0
23. A.M.E.	F.	56	20	10	19	0	—	—	—	44	36	3.8	3.5	65.0
24. E.H.M.L.	F.	44	5	5	40	+	—	4.8	—	—	—	—	—	51.0
								2.1						

1. in each columnne denotes »before treatment«.

2. in each columnne denotes »at end of treatment«.

<sup>1)</sup>  $R_a$  = resistance of afferent arterioles.

$R_e$  = resistance of efferent arterioles.

$R$  = total arteriolar resistance.

The hematocrit readings and number of red blood cells in Table 1

## Pressure and Renal Function.

Blood Pressure Mm. Hg.		Glom. Filtr. ML./Min.		Renal Plasma Flow ML./Min.		Renal Blood Flow ML./Min.		Filtration Fraction		R <sub>a</sub> <sup>1)</sup> × 1000 Mm. Hg Per ML. Per Min.		R <sub>e</sub> <sup>1)</sup> × 1000 Mm. Hg Per ML. Per Min.		R <sup>1)</sup> × 1000 Mm. Hg Per ML. Per Min.	
1.	2.	1.	2.	1.	2.	1.	2.	1.	2.	1.	2.	1.	2.	1.	2.
132/90	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
126/80	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
126/85	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
140/89	94	—	572	—	—	—	—	—	—	—	—	—	—	—	—
110/80	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
122/83	90	130	693	580	1154	951	0.13	0.22	5.10	20.61	1.63	4.03	6.73	24.64	
126/80	98	146	784	753	1450	1355	0.13	0.19	2.71	6.10	1.02	1.94	3.73	8.04	
118/78	76	83	575	626	885	894	0.13	0.13	7.86	25.66	2.37	3.72	10.23	29.38	
129/83	86	109	537	615	926	1040	0.16	0.18	10.08	8.39	2.28	2.78	12.36	11.17	
110/74	14	10	34	80	—	—	—	—	—	—	—	—	—	—	—
122/79	51	89	324	361	558	547	0.16	0.25	9.12	27.74	3.49	6.59	12.61	34.33	
appr. 115/75	18	—	176	—	275	—	0.10	—	—	—	—	—	—	—	—
91/56	40	—	200	—	256	—	0.20	—	—	—	—	—	—	—	—
114/75	73	66	399	300	712	492	0.18	0.22	2.07	26.36	2.86	5.34	4.94	31.70	
112/60	86	93	469	500	795	714	0.18	0.19	8.07	22.15	2.11	4.36	10.18	26.51	
181/95	68	90	374	329	693	531	0.18	0.27	15.11	33.13	2.99	8.96	18.10	42.09	
193/101	9	14	—	—	—	—	—	—	—	—	—	—	—	—	—
146/79	64	72	287	306	478	489	0.21	0.24	23.90	42.85	4.90	5.62	28.80	48.47	
155/90	74	95	348	324	536	491	0.21	0.29	14.87	38.28	5.24	12.66	20.11	50.94	
163/103	55	95	376	377	649	639	0.15	0.25	—	—	—	—	—	—	—
153/83	81	96	464	392	814	636	0.18	0.24	—	—	—	—	—	—	—
156/88	72	98	341	338	568	564	0.21	0.29	8.52	23.69	6.12	8.51	14.64	32.20	
appr. 173/93	85	—	appr. 300	—	appr. 540	—	appr. 0.28	—	—	—	—	—	—	—	—
183/101	77	—	440	—	830	—	0.17	—	—	—	—	—	—	—	—
163/88	106	—	410	—	733	—	0.26	—	—	—	—	—	—	—	—
173/100	44	—	—	—	—	—	—	—	—	—	—	—	—	—	—

represent the highest and lowest values observed in each case, and do not form the basis of the calculation of the renal blood flow.

The body weight, denoted in brackets, signifies the true original weight. The weight given below indicates weight after dehydration.

The blood pressures represent the mean values of the pressures during the last five days before the DCA treatment (1) and during the last five days of the DCA treatment (2).

dium chloride administration (min. a fortnight). The glomerular filtration increased in all the cases but two (cases 10 and 14). In case 14, a slight increase occurred when the DCA treatment was repeated. The mean value of the increase of the glomerular filtration was  $20.3 \text{ ml/min.} \pm 4.6$ , being therefore statistically significant. The changes of renal blood flow

Table 2.

Mean values of blood pressure before and after administration of DCA and sodium chloride in case 1—9 (part of Group I) and case 15—24 (Group II).

	Initial Blood Pressure Mm. Hg.	Final Blood Pressure Mm. Hg.	Increase of Blood Pressure Mm. Hg.	
			Systolic	Diastolic
Group I (Cases 1—9)	111/73	126/83	$14.4 \pm 2.4$	$11.3 \pm 1.8$
Group II (Cases 15—24)	123/77	169/94	$46.1 \pm 1.9$	$17.7 \pm 2.2$

are difficult to evaluate. In the majority of cases, a slight reduction took place, the mean value of the decrease being  $67.3 \text{ ml/min.} \pm 27.7$ , i. e. not significant. The filtration fraction increased in all the cases, the mean value of the increase being  $6.0 \pm 1.0$ .

5. In Group I, too, a few cases (nos. 10—14) revealed a considerable reduction in the clearance values. The following emerges from a close analysis of these cases (see page 59).

Cases 10 and 11 had renal diseases of a special type (cp. above).

Cases 12, 13 and 14 disclosed nephritis in the case history and these patients were the only ones to react with marked edema to the DCA administration.

6. In nine cases, the resistance in the afferent and efferent

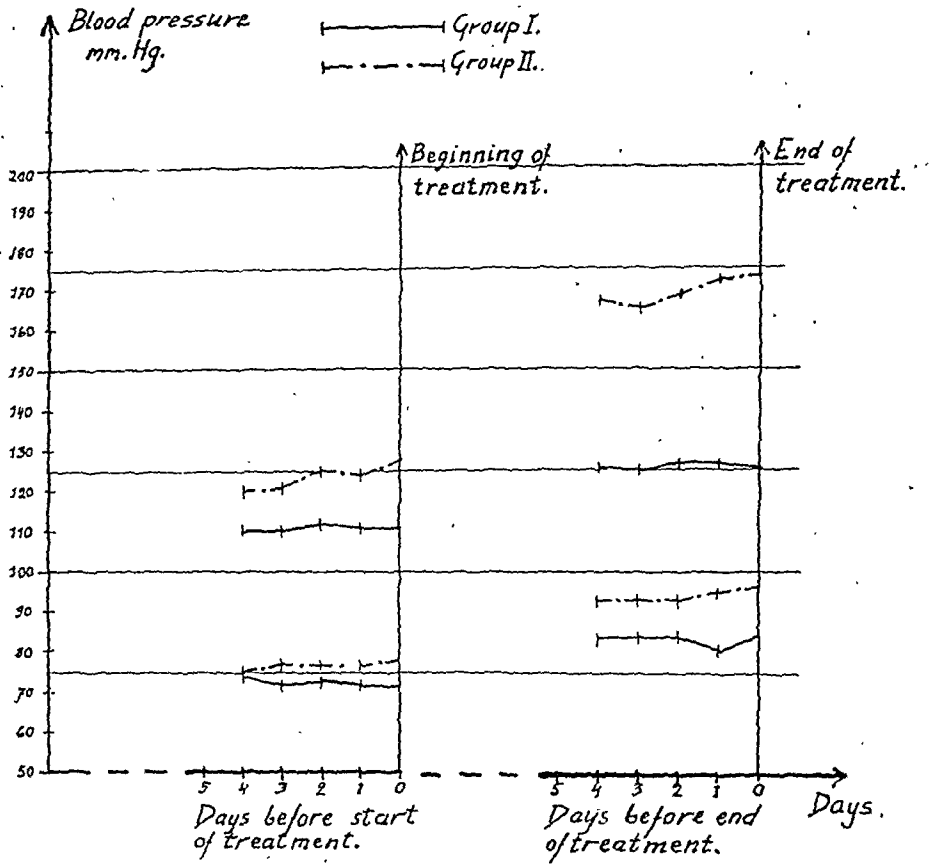


Fig. 1.

Mean blood pressure in Group I and II for each day during the last five days before treatment and during the last five days before the end of treatment.

arterioles of the kidneys, before and after DCA administration, was determined by means of formulae laid down by *Lamport* (1943). Eight of these cases showed an increased vascular resistance, as is evident from Table 1. The increase of the resistance was comparatively greater in the afferent vessels.

## DISCUSSION

The present authors have been able to establish that a small but significant increase in blood pressure can be pro-

duced in healthy subjects by DCA and sodium chloride. This increase was observed after about a week's treatment.

However, a number of patients showed a more marked increase of blood pressure. In this group, a common feature was a disordered renal function, as measured by inulin and diodrast clearances. The difference in blood pressure increase between the two groups was statistically significant. Both these groups showed similar changes in the hematocrit readings, number of red blood cells and body weight. This suggests that the increase of blood pressure was not indicative of a larger plasma volume. This is, moreover, in agreement with earlier observations (*Swingle et al.*, 1941, *Perera & Blood*, 1947, *Luft & Sjögren*, 1949 b).

Five patients with signs of severe renal damage did not react with a rise of blood pressure corresponding to that of Group II. Three of these patients, who suffered from chronic glomerulonephritis, developed marked edema a few days after the administration of DCA. Two of these were later found to have a low concentration of plasma protein, but the plasma protein concentration was not determined in the third case. A fourth patient was found, at autopsy, to have had considerable hypertrophy of the kidneys, due to marked lipoidosis. The vascular changes were only slight in this case. The fifth case probably suffered from renal amyloidosis.

In five of the ten cases in this group that reacted with a rise in blood pressure, a chronic glomerulonephritis was present. In the other cases the low renal blood flow could not be explained, but it is possible that a chronic glomerulonephritis was present in some of them in spite of the absence of typical urinary findings. The glomerular filtration of Group II was significantly lower than the normal value. The results obtained in the present investigation in the human subject correspond to those arrived at by *Knowlton et al.* (1946) and *Loeb et al.* (1949) in rats in which they had produced renal damage by means of a nephrotoxic serum.

Previously the effect of DCA and sodium chloride on renal function in man has only been examined in isolated cases of

Addison's disease, some of which showed a rise of glomerular filtration after DCA administration (*Talbott et al.*, 1943, *Lüft & Sjögren*, 1949 c). In the present material, an increased glomerular filtration and filtration fraction was found in healthy subjects as well as in those suffering from disordered renal function. The increase was not particularly large in the cases that manifested hypotension. There was a tendency to reduced renal blood flow in the present material after DCA administration. The change of renal blood flow was independent of the increase of blood pressure.

It is probable that a connection existed between the hypertension produced in Group II and the disordered renal function found in these cases. It is, of course, impossible to decide whether the increase of blood pressure had any direct pathogenetic connection with the reduced renal blood flow occurring in these cases, or with some other injury to the function of the kidney.

In all likelihood, the increase of blood pressure was due to increased peripheral vascular resistance. However, since the total peripheral vascular resistance was not determined, this problem cannot be regarded as solved. The increase of the resistance in the arterioles of the kidneys, calculated by us, cannot be taken as proof of a general increase of peripheral resistance. The increase of arteriolar resistance may be interpreted as a sign of the renal reaction to an increased blood pressure, irrespective of the manner in which this rise of blood pressure was induced (*Lamport*, 1942, 1943).

## SUMMARY

1. Patients were treated with DCA and sodium chloride for at least two weeks. In one group of subjects with a mean initial pressure of 123/77 mm Hg., a final pressure of 169/94 mm Hg. was obtained. This group was characterized by reduced values of inulin and diodrast clearances. The mean value of the initial pressure in the other patients equalled

111/73 mm Hg., the final pressure being 126/83 mm Hg. The difference in blood pressure increase between the two groups was statistically significant as was also the rise of blood pressure in each group.

2. Both groups of patients showed an equal reduction in the hematocrit readings, number of red blood cells, and a comparable increase of body weight.

3. A group of five patients with renal damage did not react with an increase of blood pressure. Three of them developed marked edema a few days after the administration of DCA. One patient had marked renal lipoidosis, another renal amyloidosis.

4. The inulin and diodrast clearances were studied in twelve cases before and after the administration of DCA and sodium chloride. The glomerular filtration increased by 20.3 ml/min.  $\pm$  4.6, and the filtration fraction by  $6.0 \pm 1.0$ . In the majority of cases, a slight decrease of renal blood flow occurred, but the decrease was not statistically significant.

5. DCA produced an increase of the peripheral renal vascular resistance, calculated according to Lampport's formulae. This increase was proportionately greater in the afferent than in the efferent arterioles.

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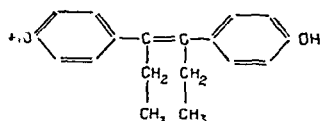
## SYNTHETIC OESTROGENIC SUBSTANCES A COMPARATIVE STUDY ON THEIR EFFECTIVENESS IN WOMEN

BY

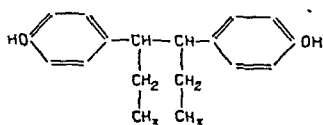
ÅKE B. V. RYDÉN

A real understanding of the relative effectiveness of the various — natural or synthetic — oestrogenic substances in women is of great importance for the establishment of an adequate and well founded oestrogenic therapy.

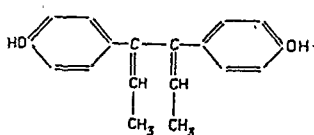
During the years 1936—38 *Dodds* and coworkers described a series of various synthetic derivatives with more or less marked oestrogenic properties. The three most effective compounds have been called stilboestrol, dienoestrol and hexoestrol.



Stilboestrol



Hexoestrol



Dienoestrol

The three compounds mentioned above, possessed not only all the characteristic properties of the naturally occurring oestrogenic substances, but — according to the experiments of *Dodds* — they showed a greater potency than the natural oestrogens, when administered subcutaneously, hexoestrol

being 2.8-times, dienoestrol 2.3-times and stilboestrol twice as active as oestrone. These experiments of *Dodds et al.* have been carried out on rats, with the assay-technique by *Allen and Doisy*. The same results were obtained by *Emmens* (1938), using experimental conditions very similar to those of *Dodds*. These experiments were further confirmed by *Munro & Kosin* (1946). Compared on the basis of oviduct stimulation in chicks they found that given orally the decreasing order of potency was as follows: hexoestrol, dienoestrol and stilboestrol.

On the other hand *Dodds et al.* (1939) demonstrated that stilboestrol and hexoestrol assayed on immature rats by the »increase in uterine weight«-method, showed the same potency. It has been shown furthermore by *Kreitmaier & Sieckmann* (1939), *Pedersen-Bjergaard* (1939) and *Andersen* (1941) that assays of both natural and synthetic oestrogens produce inconstant and inconsistent results due to differences in animal species, methods of assay and routes of administration.

It is nowadays generally understood that the naturally occurring oestrogenic substances undergo an enzymatic inactivation, which takes place in the liver (*Zondek*, 1934, *Golden & Sevringhaus*, 1938, *Israel*, *Meranze & Johnston*, 1937, *Fels*, 1939, and others). The extent of this inactivation was estimated by *Pedersen-Bjergaard* (1939) as being as high as 90 per cent. The fact, that synthetic oestrogens are involved to a much smaller extent in this inactivation, offers a favourable opportunity for successful peroral oestrogenic therapy. It has been claimed by *Pedersen-Bjergaard et al.* (1947) that perorally administered hexoestrol possesses only one-tenth of the activity of stilboestrol and hardly one-tenth of that of dienoestrol when assayed on rats by the vaginal smear technique. This seems to indicate that the liver inactivates hexoestrol to a considerably greater extent than the other two synthetic compounds. In contrast to this observation, it has been reported by *Segaloff* (1944) that both stilboestrol and hexoestrol are inactivated to the same extent by the liver.

As the results obtained in animal experiments could not

be directly applied to the conditions in women, several experiments were carried out in order to obtain some information concerning the relative potency of these synthetic oestrogens in women.

Thus *Bieren & Compton* (1942) could favourably influence the vasomotor disturbances, accompanying menopausal symptoms by the peroral administration of stilboestrol and hexoestrol, and estimated by this method that stilboestrol was about ten times as active as hexoestrol. Using the same technique *Greenhill* (1942) calculated a ratio in potency 1:2.5 to 1:5 in favour of stilboestrol. Practically the same results have been obtained by *Crotty, Schloss & Lyford* (1943) under very similar experimental conditions. According to the experiments of *Harding* (1946) stilboestrol dipropionate was 3—6 times greater in activity than hexoestrol when administered to women suffering from menopausal disturbances.

By the administration of oestrogenic substances proliferation can be elicited in a previously inactive endometrium; the interruption of this treatment generally results in a uterine bleeding, the so-called »withdrawal bleeding«. Investigating the minimal effective dose of various oestrogenic substances inducing such a bleeding in castrated women, *Allen* (1944) found that stilboestrol was 3.5 times as active as hexoestrol. *Ferin* (1941) showed in a castrated woman, that in the case of stilboestrol the »endometrial threshold dose« for eliciting withdrawal bleeding (called by him »woman-unit«) lay between 0.1 to 0.2 mg. per day, when administered during a period of 14 days, whereas the corresponding values for dienoe-strol and hexoestrol were 0.1—0.25 and 1.0—2.0 mg. per day. The same author (1946), in another experiment carried out on a woman castrated surgically during sexual maturity, found that both stilboestrol and dienoe-strol were equally active and considerably superior to hexoestrol. In this experiment endometrial biopsies were also examined, without, however, any special comparison of the degree of proliferation.

Besides this type of human bioassay, mentioned above,

some other methods of assay are also described in the literature. Thus *Barnes* (1942) based her investigations on the capacity of the synthetic oestrogens to suppress lactation during the puerperium, and found that stilboestrol — although more potent than hexoestrol — exhibits only one-tenth of the activity of dienoestrol. In another attempt to find a suitable human bioassay method, *Bieren & Compton* (1942) found that hexoestrol was more active than stilboestrol in the treatment of gonorrhoeal vulvo-vaginitis in children.

However, all of these human bioassay methods require a critical examination and above all some well-defined requirements are needed for a suitable and reliable assay method.

Thus in assays of oestrogenic substances in women — as in animal experiments — it is absolutely necessary to eliminate — as far as possible — the body's own oestrogenic hormone production. This criterion can be said to be achieved only after the surgical removal of the ovaries. Secondly, the organ or tissue, in which the changes are mirrored ought to react to the administration of synthetic substances in the same manner as to the normally occurring »intrinsic« oestrogens, i. e. the experiment must be carried out on a person castrated during sexual maturity. Finally the chosen method of assay must be based upon changes, definitely brought about by the oestrogens, and varying with the dose administered and which easily could be recorded by the investigator.

In view of these criteria the beneficial effect on menopausal vasomotor symptoms, — greatly conditioned by psychological factors — the suppression of lactation during puerperium and the successful results in the treatment of gonorrhoeal vulvo-vaginitis cannot serve as reliable methods of assay. This view is further supported by the experiments of *Weaver* (1946), in which menopausal disturbances were favourably influenced by the administration of peanut oil, regardless of its oestrogen content. In this respect the data of *Bennet* (1942) are also of interest; he states that no definite relation can be established between the doses of oestrogens

necessary for the suppression of menopausal disturbances and those necessary to bring about objective changes (for instance in the vaginal smear).

An assay technique, based upon the threshold values for eliciting a withdrawal bleeding should probably offer a more reliable method; but in this case it must first be demonstrated with certainty that these bleedings occur invariably with the same degree of proliferation in a given well defined endometrium. As far as I know, up till now no such findings have been published.

Of course, a human bioassay method could possibly be based upon the changes in the vaginal smear, occurring after the administration of various oestrogenic substances, as demonstrated first by *Stockhard & Papanicolaou* (1917); but in contrast to the conditions in rodents, the comparison of the vaginal changes in women is difficult and makes the assay very unreliable.

Among the conceivable bioassay methods in the human thus there remains only one which offers the greatest reliability and objectiveness, and this is based on the proliferative changes in the endometrium, brought about by the oestrogens in women castrated during sexual maturity.

However, the very limited number of patients, surgically castrated during sexual maturity, available for experimental purposes, could not enable us to assay every substance on one group of patients, and we were forced to administer the substances to be assayed in a given order to each experimental subject.

## MATERIAL

Having demonstrated by endometrial biopsy the presence of an inactive endometrium of a castration-type, in six women, aged 27—48 years, surgically castrated during sexual maturity a compound of the stilboestrol type (Stilbol), dienoestrol type (Synsteron) and hexoestrol type (Novostrol) was admi-

nistered orally.<sup>1)</sup> The daily dose of the preparations in the case of the first two patients was 5 mg. The oestrogenic effect on the endometrium was controlled by endometrial biopsies at weekly intervals. At the same time, the effect on the subjective menopausal disturbance, changes in the vaginal mucosa (showing invariably more or less definite atrophy at the beginning of the experiment), the growth of the myometrium (measured by uterine sound), together with the establishment of uterine bleeding were followed. Between the various experimental periods there was an interval of at least three weeks, when no oestrogen-treatment was given. As this study was confined to the proliferative changes of the endometrium, the administration of oestrogens was in no case followed by the administration of progesterone or any other substance possessing a corpus luteum effect.

In the course of a preliminary examination of the endometrial biopsies obtained from the first two cases it became obvious, however, that the hexoestrol-preparation possessed a lower activity as compared to the others. Consequently, in order to make the comparison between the various substances easier, the daily doses were modified as follows: stilboestrol, 4 mg., dienocetrol, 4 mg. and hexoestrol, 8 mg. This dosage-scheme was continued during the whole course of the experiment.

At the end of the experiment the endometrial biopsies were classified microscopically with regard to the extent of proliferation present, and corresponding biopsies were chosen for comparison.

The results are summarized in Tables 1—6.

*Rate of proliferation:*

O = material insufficient for making a diagnosis

P<sub>1</sub> = inactive endometrium

P<sub>2</sub> = early proliferation

P<sub>3</sub> = completely developed proliferation

P<sub>4</sub> = hyperproliferation

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<sup>1)</sup> I am greatly indebted to AB Leo, Hålsingborg, and AB Hässle, Hässleholm, for their kindness of supplying me with the preparations used throughout these experiments.

Case 4.

S. B. Age: 45. Time from castration to beginning of the experiments: 2½ months.  
Administration of oestrogens: Stilboestrol 5 mg. orally daily

Dienoestrol 5 » » » »

Hexoestrol 5 » » » »

Date of investigation 1946	Oestrogens administered from the beginning of the experiment (mg.)			Menopausal symptoms	Atrophy of the vaginal mucosa	Uterine size in cm (measured by sound)	Date of last dose of oestrogens	Uterine bleeding	Histological report	Degree of proliferation equal to that of spec.
	Stilboestrol	Dienoestrol	Hexoestrol							
23/9	0			+++	+	7			O	
30/9	35			+	0	8½			P <sub>3</sub>	26/11, 23/1
7/10	70			0	0	9			P <sub>3</sub>	
12/10	95			0	0	9	12/10	14/10—23/10	P <sub>4</sub>	
6/11			0	+++	0	7			O	
14/11			40	++	0	7¾			P <sub>2</sub>	
20/11			70	0	0	8			P <sub>3</sub>	
26/11			95	0	0	8			P <sub>3</sub>	30/9, 23/1
4/12			140	0	0	8			P <sub>2</sub>	
12/12			180	0	0	8	12/12	14/12—19/12	P <sub>2</sub>	
14/1 1947		0		+++	+	7			O	
23/1		45		0	0	8			P <sub>3</sub>	30/9, 26/11
29/1		75		0	0	8			P <sub>4</sub>	
6/2		115		0	0	8			P <sub>4</sub>	
13/2		150		0	0	9	13/2	14/2—21/2	P <sub>4</sub>	



## Case 2.

K. G. Age: 39. Time from castration to beginning of the experiments: 1½ months.  
 Administration of oestrogens: Stilboestrol 5 mg. orally daily  
 Dioestrol 5 » » » »  
 Hexoestrol 5 » » » »

Date of investigation	Oestrogens administered from the beginning of the experiment (mg.)			Menopausal symptoms	Atrophy of the vaginal mucosa	Uterine size in cm (measured by sound)	Date of last dose of oestrogens	Uterine bleeding	Histological report	Degree of proliferation equal to that of spec.
	Stilboestrol	Dioestrol	Hexoestrol							
1946										
30/11		0		+	+	6½			P <sub>1</sub>	
4/12		45		0	0	7¾			P <sub>2</sub>	24/2, 29/4
16/12		80		0	0	8	23/12	28/12—1/1	P <sub>3</sub>	
23/12		115		0	0	8			P <sub>3</sub>	
1947										
3/2			0	+	+	6½			P <sub>1</sub>	
10/2			35	+	+	8½			P <sub>2</sub>	
17/2			70	0	0	8½			P <sub>3</sub>	16/12, 29/4
24/2			105	0	0	8½			P <sub>3</sub>	
3/3			140	0	0	8½	10/3	15/3—16/3	P <sub>2</sub>	
10/3			175	0	0	8				
22/4	0			+	+	6½			O	16/12, 24/2
29/4	35			0	0	9			P <sub>3</sub>	
6/5	70			0	0	9	13/5	19/5—24/5	P <sub>4</sub>	
13/5	105			0	0	9			P <sub>4</sub>	

## Case 3.

I. L. Age: 35. Time from castration to beginning of the experiments: 3 months.

Administration of oestrogens: Stilboestrol 4 mg. orally daily

Dienoestrol 4 » » »

Hexoestrol 8 » » »

Date of investigation 1947	Oestrogens administered from the beginning of the experiment (mg.)			Menopausal symptoms	Atrophy of the vaginal mucosa	Uterine size in cm (measured by sound)	Date of last dose of oestrogens	Uterine bleeding	Histological report	Degree of proliferation equal to that of spec.
	Stilboestrol	Dienoestrol	Hexoestrol							
19/3		0		+	+	5½			O	
26/3		56		+	+	6			P <sub>3</sub>	
2/4		112			0	7			P <sub>3</sub>	
9/4		168			0	7			P <sub>4</sub>	18/6, 28/8
16/4		224			0	7	16/4	18/4—22/4	P <sub>4</sub>	
28/5		0		+	+	5½			O	
4/6		28			0	7			P <sub>2</sub>	
11/6		56			0	7½			P <sub>3</sub>	
18/6		84			0	7½			P <sub>4</sub>	9/4, 28/8
21/6		96			0	7½	21/6	23/6—27/6	P <sub>4</sub>	
20/8	0			+	+	5½			O	
28/8	32				0	7			P <sub>4</sub>	9/4, 18/6
4/9	60				0	7			P <sub>3</sub>	
9/9	80				0	7	9/9		P <sub>4</sub>	

## Case 4.

M. F. Age: 38. Time from castration to beginning of experiments: 5 months.

Administration of oestrogens: Stilboestrol 4 mg. orally daily

Dienoestrol 4 » » » »

Hexoestrol 8 » » » »

Date of investigation 1946	Oestrogens administered from the beginning of the experiment (mg)			Menopausal symptoms	Atrophy of the vaginal mucosa	Uterine size in cm (measured by sound)	Date of last dose of oestrogens	Uterine bleeding	Histological report	Degree of proliferation equal to that of spec.
	Stilboestrol	Dienoestrol	Hexoestrol							
8/3		0		+++	++	6			O	
15/3		28		++	0	7 <sup>3</sup> / <sub>4</sub>			P <sub>2</sub>	14/6
22/3		56		0	0	8	30/3	1/4-5/4	P <sub>3</sub>	26/4
30/3		88		0	0	8			P <sub>4</sub>	
19/4	0			+++	++	6			P <sub>1</sub>	30/3
26/4	28			0	0	9			P <sub>4</sub>	
3/5	56			0	0	8 <sup>1</sup> / <sub>2</sub>			P <sub>4</sub>	
10/5	84			0	0	8 <sup>1</sup> / <sub>2</sub>	10/5	12/5-20/5	P <sub>4</sub>	
31/5			0	+	++	6			P <sub>1</sub>	
7/6			56	0	0	7 <sup>3</sup> / <sub>4</sub>			P <sub>2</sub>	22/3
14/6			112	0	0	7 <sup>3</sup> / <sub>4</sub>			P <sub>3</sub>	
21/6			168	0	0	7 <sup>1</sup> / <sub>2</sub>	21/6		P <sub>3</sub>	

*Case 5.*  
H. A. Age: 27. Time from castration to beginning of experiments: 1½ month.  
Administration of oestrogens: Stilboestrol 4 mg. orally daily  
Dienoestrol 4 » » » »  
Hexoestrol 8 » » » »

Date of investigation	Oestrogens administered from the beginning of the experiment (mg.)			Menopausal symptoms	Atrophy of the vaginal mucosa	Uterine size in cm (measured by sound)	Date of last dose of oestrogens	Uterine bleeding	Histological report	Degree of proliferation equal to that of spec.
	Stilboestrol	Dienoestrol	Hexoestrol							
1918										
18/3	0			++	++	6½			O	
25/3	28			0	+	7½			P <sub>3</sub>	
1/4	56			0	0	8			P <sub>4</sub>	14/5, 25/6
8/4	84			0	0	7¾	8/4	15/4—23/4	P <sub>4</sub>	
29/4			0	+	++	6½			P <sub>1</sub>	
7/5			72	0	+	7			P <sub>2</sub>	
14/5			120	0	0	7			P <sub>4</sub>	1/4, 25/6
20/5			168	0	0	8	20/5	20/5—29/5	P <sub>4</sub>	
4/6		0		++	++	6½			P <sub>1</sub>	
11/6		28		0	0	7½			P <sub>2</sub>	
18/6		56		0	0	7½			P <sub>3</sub>	
25/6		84		0	0	8	25/6	5/7—12/7	P <sub>4</sub>	1/4, 14/5

Case 6.

I. S. Age: 48. Time from castration to beginning of experiments: 6½ months.

Administration of oestrogens: Stilboestrol 4 mg. orally daily

Dienoestrol 4 » » » »

Hexoestrol 8 » » » »

Date of investigation 1948	Oestrogens administered from the beginning of the experiment (mg.)			Menopausal symptoms	Atrophy of the vaginal mucosa	Uterine size in cm (measured by sound)	Date of last dose of oestrogens	Uterine bleeding	Histological report	Degree of proliferation equal to that of spec.
	Stilboestrol	Dienoestrol	Hexoestrol							
9/4			0	+	+	+	5½		O	
16/4			56	+	+	0	7		P <sub>2</sub>	
23/4			112	+	0	0	7¼		P <sub>4</sub>	11/6, 6/8
30/4			168	+	0	0	7	2/5—6/5	P <sub>4</sub>	
4/6	0			+	+	+	6		P <sub>1</sub>	
11/6	28			+	0	0	7¼		P <sub>4</sub>	23/4, 6/8
18/6	56			0	0	0	7½		P <sub>4</sub>	
25/6	84			0	0	0	7½	4/7—11/7	P <sub>4</sub>	
23/7		0		+	+	+	6		P <sub>1</sub>	
30/7		28		+	0	0	7½		P <sub>3</sub>	
6/8		56		0	0	0	8		P <sub>4</sub>	23/4, 11/6
13/8		84		0	0	0	7¾	17/8—24/8	P <sub>4</sub>	

<i>Atrophy of the vaginal mucosa:</i>	<i>Grades of subjective menopausal symptoms:</i>
+++ = very definite atrophy	+++ = severe menopausal symptoms
++ = moderate atrophy	++ = moderate menopausal symptoms
+ = slight atrophy	+ = slight menopausal symptoms
0 = normal vaginal mucosa	0 = no menopausal symptoms

With regard to their ability of producing endometrial proliferation, it has been shown that the effect of 1.0 mg. stilboestrol corresponds to the following amounts of dienoeestrol or hexoeestrol, investigated in each of the six patients:

Case:	Stilboestrol:	Dienoestrol:	Hexoestrol:
1	1.0	1.3	2.7
2	1.0	2.3	3.0
3	1.0	2.6	5.2
4	1.0	3.1	6.2
5	1.0	1.5	2.1
6	1.0	2.0	4.0
Average:	1.0	2.1	3.9

## DISCUSSION

In castrated women the three synthetic oestrogenic substances investigated behave qualitatively in the same manner in producing the following four effects:

1. Concerning the vasomotor symptoms following castration, it can be stated that they are suppressed by oestrogenic medication. In this respect stilboestrol is slightly more effective than dienoeestrol, but both are highly superior to hexoeestrol, as is excellently demonstrated in case 6, where not even daily doses of 8 mg. hexoeestrol could suppress the vasomotor symptoms, even though it was administered continuously for three weeks.

2. Under the influence of oestrogenic substances, the vaginal mucosa — more or less atrophic after castration — begins to grow, and assumes a normal appearance and consistency. This change takes place so soon that — at the dose levels administered — there is no opportunity for differentiating the relative activity of the three substances. This change takes place very soon, frequently before subjective symptoms have been relieved.

3. All the three compounds investigated cause an increase

in the size of the myometrium, as measured by the uterine sound. In this respect stilboestrol and dienioestrol have about the same activity, and both are more active than hexoestrol. The relative inaccuracy of this method, however, does not allow any more exact conclusions.

4. All the three compounds produce a proliferation in the inactive endometrium, which — with sufficiently high doses — may result in a hyperproliferation. In these series of investigations, however, where oestrogenic treatment is always discontinued after a period of 3—5 weeks, it never reaches that degree of proliferation, which could correspond to a fully developed cystic-glandular hyperplasia. In this assay stilboestrol showed the greatest activity, being twice as active as dienioestrol and four times as active as hexoestrol.

In order to obtain a fully developed proliferation the following amounts of the various substances were needed: stilboestrol, < 28—35 mg., dienioestrol, 28—80 mg. and hexoestrol, 70—112 mg. respectively.

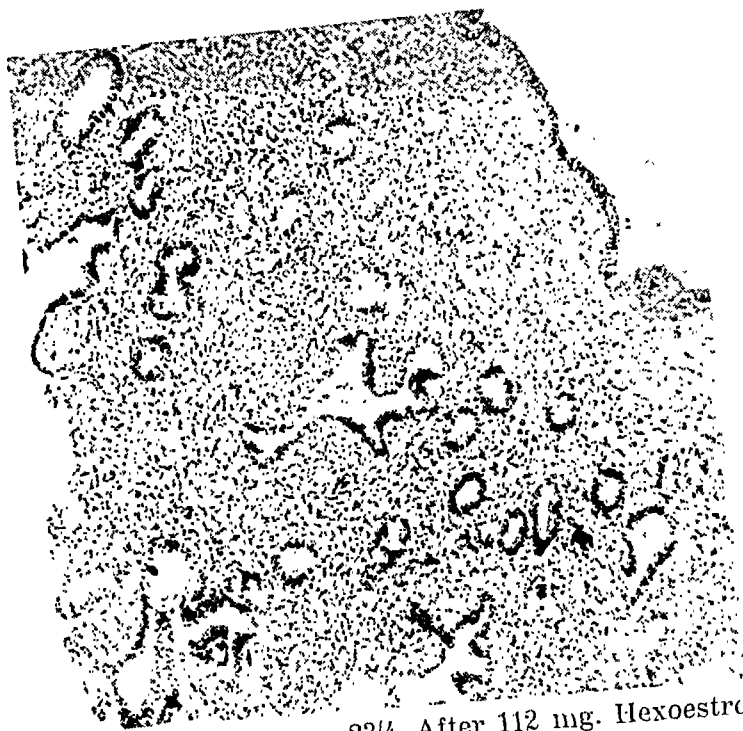
In a previous paper (1947) it was stated that the oestrogen-dose, required for full endometrial proliferation, has to be increased in relation to the time which had elapsed between oophorectomy and treatment. All the present cases were castrated only a short time before treatment. Thus they throw no additional light on the question.

The investigation of the changes in the endometrial stroma produced by the oestrogenic substances during proliferation, was outside the scope of this paper and therefore it will not be discussed here. These changes are intended to be specially investigated, and the results will be reported in a later paper.

Toxic side-effects caused by oestrogenic substances administered in the course of this investigation were not particularly marked. Initial nausea occurred in a few cases, but disappeared during the continued drug-administration. Vomiting never occurred. It would appear that among the substances investigated stilboestrol caused rather more toxic side-effects than dienioestrol, while hexoestrol in the doses administered never caused any toxic effects. In all the patients the administration of the drugs was carried out as intended without difficulty.



*Fig. 1. Case 6. Biopsy 9/4 before the beginning of experiment:  
inactive mucosa. 150 X.*



*Fig. 2. Case 6. Biopsy 23/4. After 112 mg. Hexoestrol:  
hyperproliferation. 60 X.*





*Fig. 3. Case 6. Biopsy 11/6. After 28 mg. Stilboestrol: hyperproliferation. 60 X.*



*Fig. 4. Case 6. Biopsy 6/8. After 56 mg. Dienoestrol: hyperproliferation. 60 X.*

## SUMMARY

1. Three synthetic oestrogenic substances, stilboestrol, dienoestrol and hexoestrol given orally have been investigated for their relative effect in women, surgically castrated during sexual maturity. Their relative potency showed a definite relation with regard to the four different methods of assay, used.

2. The vasomotor disturbances, following castration, were favourably influenced by all the synthetic oestrogens administered. Hexoestrol, however, showed a considerably smaller activity as compared with stilboestrol or dienoestrol.

3. The vaginal mucosa, showing signs of atrophy due to castration, began to develop, and soon assumed a normal appearance as a result of the administration of synthetic oestrogens.

4. The three compounds investigated induced a growth of the uterine myometrium. In this respect hexoestrol showed the slightest effect.

5. The substances examined induced a proliferation in the castration-type endometrium, the following amounts being necessary to obtain a completely proliferated endometrium: stilboestrol, < 28—35 mg., dienoestrol, 28—80 mg. and hexoestrol, 70—112 mg. respectively.

6. With regard to the proliferation-inducing capacity of the oestrogens investigated, it has been shown that *the effect of 1 mg. stilboestrol corresponds to that of 2 mg. dienoestrol and 4 mg. hexoestrol.*

Finally I should like to express my indebtedness to professors A. Westman and A. Sjövall for their advices and invaluable suggestions.

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THE INFLUENCE OF HYPOPHYSECTOMY AND  
OF SUBSEQUENT TREATMENT WITH  
CHORIONIC GONADOTROPHIN ON FOLLICLES  
OF DIFFERENT SIZE IN THE OVARY  
OF THE RAT

BY

F. J. A. PAESI

INTRODUCTION

A survey of the literature concerning the influence of gonadotrophins and sex hormones on the ovarian follicle reveals much that remains to be elucidated. The largest follicles fail to develop in hypophysectomized animals, but little is known with certainty of the hormonal requirements of small follicles and primordial ova, or about factors governing atresia. Several investigators counted follicles under various conditions, but only the results obtained by *Swezy* (1933) bear reference to the problem dealt with in this paper. She was the first to count primordial ova and small follicles in *hypophysectomized* animals (rats); the combined number *increased* after hypophysectomy. Three months later medium-sized follicles still showed an »abundance of mitoses«. Our own findings were in good agreement with those of *Swezy*.

We present data on the number of follicles of different size after hypophysectomy and after subsequent treatment with chorionic gonadotrophin\*). In the ovary of the hypophys-

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\*) »Pregnyl«, kindly supplied by Organon Ltd.

ectomized rat, this hormone causes only interstitial hypertrophy and production of androgen (*Paesi & Gaarenstroom*, 1943). It was our purpose to determine whether a close examination would reveal some effect on the follicles.

## MATERIAL AND METHODS

In eight young rats (61—72 gm.) the hypophysis and the right ovary were removed. Four rats were then treated with 5 I. U. of Pregnyl daily for one week. The others received saline. On the eighth day the animals were killed and the left ovary and the sella turcica removed. The histological technique was as described previously. Section-thickness: 8  $\mu$ . No remnants of the hypophysis were found in the sellae. By using the right ovary of every animal as a control for the left one, we were able to arrive at certain conclusions which otherwise would have required a greater number of animals. We measured all follicles, using a micrometer-eyepiece. The mean of the two largest diameters found to cross at right angles in one and the same section was determined. In most cases this occurred in those sections where the ovocyte and its nucleus were visible; there was therefore little danger of the follicles being counted more than once. The diameters were divided into size-groups differing by 10  $\mu$ . This method is somewhat rough-and-ready, but the results were sufficiently reliable to allow of some preliminary statements (see Fig. 1). The smallest elements counted were the smallest primordial follicles, i. e., ovocytes, surrounded by one layer of granulosa cells. The primordial ova, i. e., ovocytes surrounded by flat cells, were not counted. A follicle was called »subatretic«, when the nuclear chromatin of part of the granulosa cells was pycnotic or granular or when the egg nucleus could no longer be discerned. Advanced stages of atresia could not be considered, as they involve deformations of the follicles, which make it impossible to determine their original size. A follicle was labelled »cavity-containing« when liquor was clearly visible.

In a second experiment, originally serving another purpose, one group of animals received saline and another chorionic gonadotrophin (5 I. U. daily) between the 8th and the 17th day following hypophysectomy. All these animals had already been treated with chorionic gonadotrophin during days 1 to 7. Only every tenth section was available. The results of this experiment therefore do not possess the same significance as those of its predecessor; they are used as corroborative evidence only (Fig. 4).

In the following sections we deal consecutively with the total number of follicles, the smallest and largest follicles, atresia, the follicles of intermediate size, and cavity-formation. In every section we begin with the changes following hypophysectomy (A), and then turn to those occurring during the ensuing treatment with chorionic gonadotrophin (B).

## RESULTS

### *The ovary weight and the total number of follicles.*

A. The mean ovarian weight fell in the first week after hypophysectomy from 5.5 mg. (av. of 6, 5.5, 5 and 6 mg.) to 4 mg. (av. of 6, 3, 2.5 and 4 mg.) as a result of atresia of large follicles and atrophy of the interstitial tissue. Eight days after hypophysectomy the total number of follicles still amounted to 94 per cent of the original value (av. of 92, 84, 97 and 96 per cent). This apparently small change hides the following opposing tendencies: (1) a large increase in the number of the smallest follicles (23—32  $\mu$ ), (2) an almost complete disappearance of follicles larger than 300  $\mu$ , (3) a slight increase in the number of follicles of intermediate size.

B. As Pregnyl caused interstitial hypertrophy, the ovaries maintained their weight (right ovaries: 9, 6, 6 and 4 mg.; left ovaries 9, 6, 7 and 5 mg.). The total number of follicles decreased to 91 per cent of the original value (av. of 76, 94, 94 and 99 per cent). The distribution of the follicles over the various size-groups has much in common with that found in A (Fig. 1). There are, however, two points of difference: (a) the increase in the number of the smallest follicles is less marked than in A, (b) the largest follicles appear to be smaller than in A. These differences are also demonstrated in Fig. 2 R and 2 L, the two graphs giving the number of follicles found in successive size-groups as a percentage of the total number.

Unlike Fig. 1, Fig. 2 presents together in one section the data on the right ovaries of both groups, in another those pertaining to the left ovaries of both groups. This was permissible because the values found for the right

ovaries, i. e., the initial values, proved to be almost identical in the two groups. It seems to us that this is evidence for the reliability of our countings.

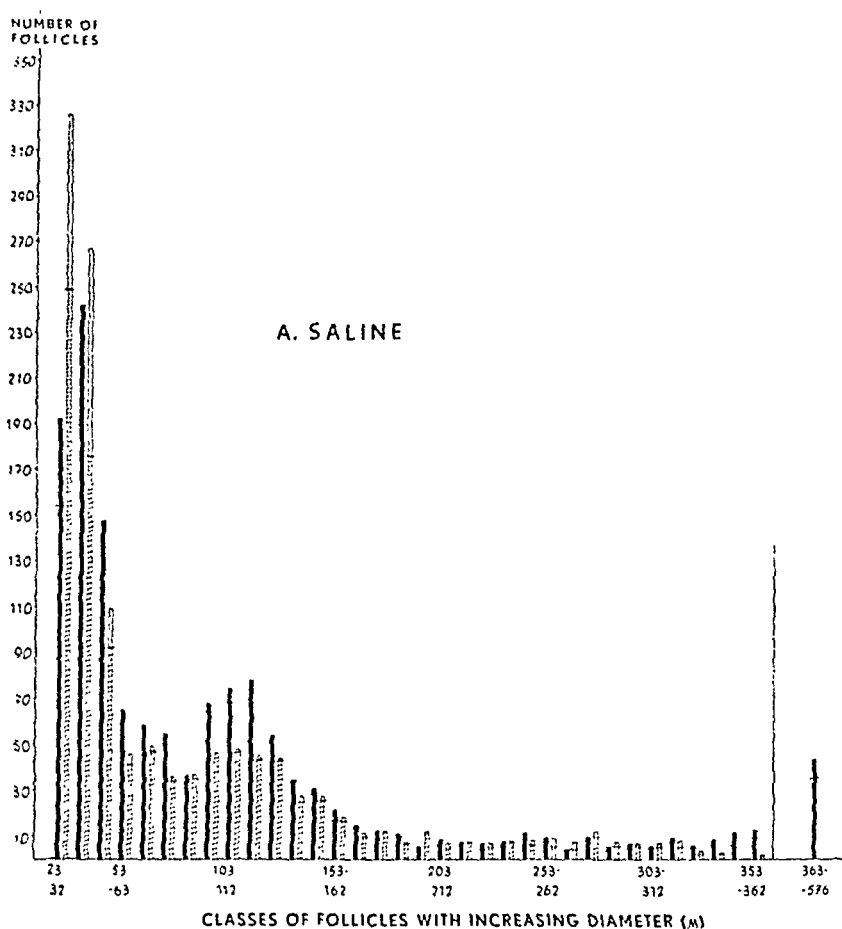


Fig. 1 A.

*The smallest follicles (23—32  $\mu$ ).*

A. The increase in the number of these follicles after hypophysectomy amounted on the average to 71 per cent. There was a marked increase in three of the four animals (by 115, 203 and 82 per cent). In the fourth the number decreased by 7 per cent. This deviation suggests that the real mean is higher than 71 per cent. To check our find-

ings we counted the smallest follicles again in all ovaries and found the same trend in each case.

Several explanations seem possible, and these are referred to in the discussion. Even at this stage, however,

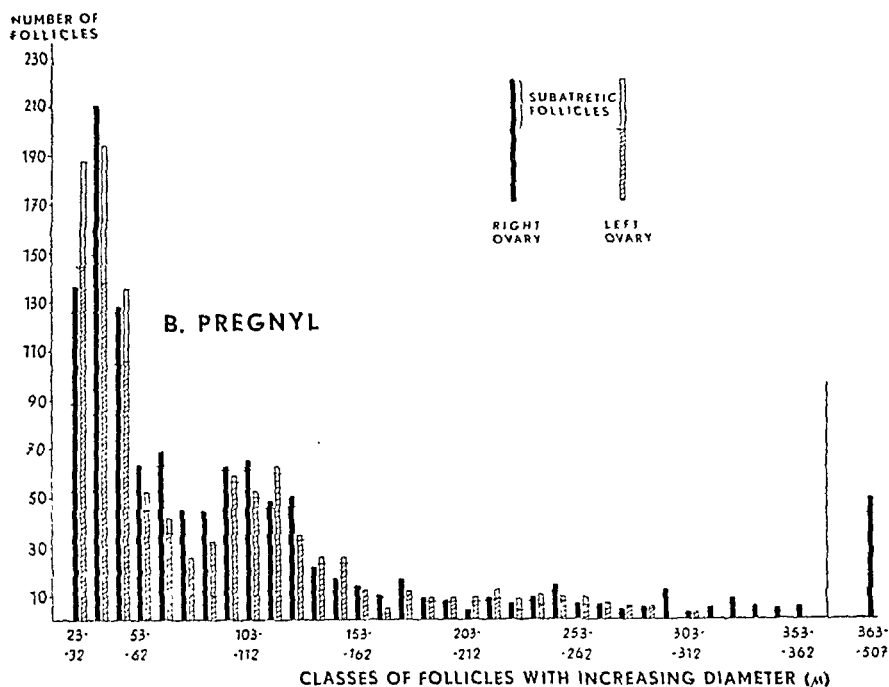


Fig. 4 B.

Number of follicles in the ovaries of semi-spayed (r) hypophysectomized young rats treated with: A. Saline, B. Pregnyl (5 I. U. daily) during 7 days. Four rats in each group. Left ovary removed on the 8th day.

Black columns: numbers before treatment (right ovaries).

Shaded columns: numbers after treatment (left ovaries).

we conclude that the formation of the first layer of granulosa-cells around the ovum is less markedly dependent on the hypophysis than the subsequent development of the young follicle.

It is noteworthy (cf. Fig. 4) that the number of the smallest follicles also increased between the 8th and the 17th day after hypophysectomy. There was a marked increase in three of four of the experimental animals (4—



19, 10—23, 10—22), in the fourth a slight decrease (18—14). The treatment with chorionic gonadotrophin during the first week will have kept the number of the small

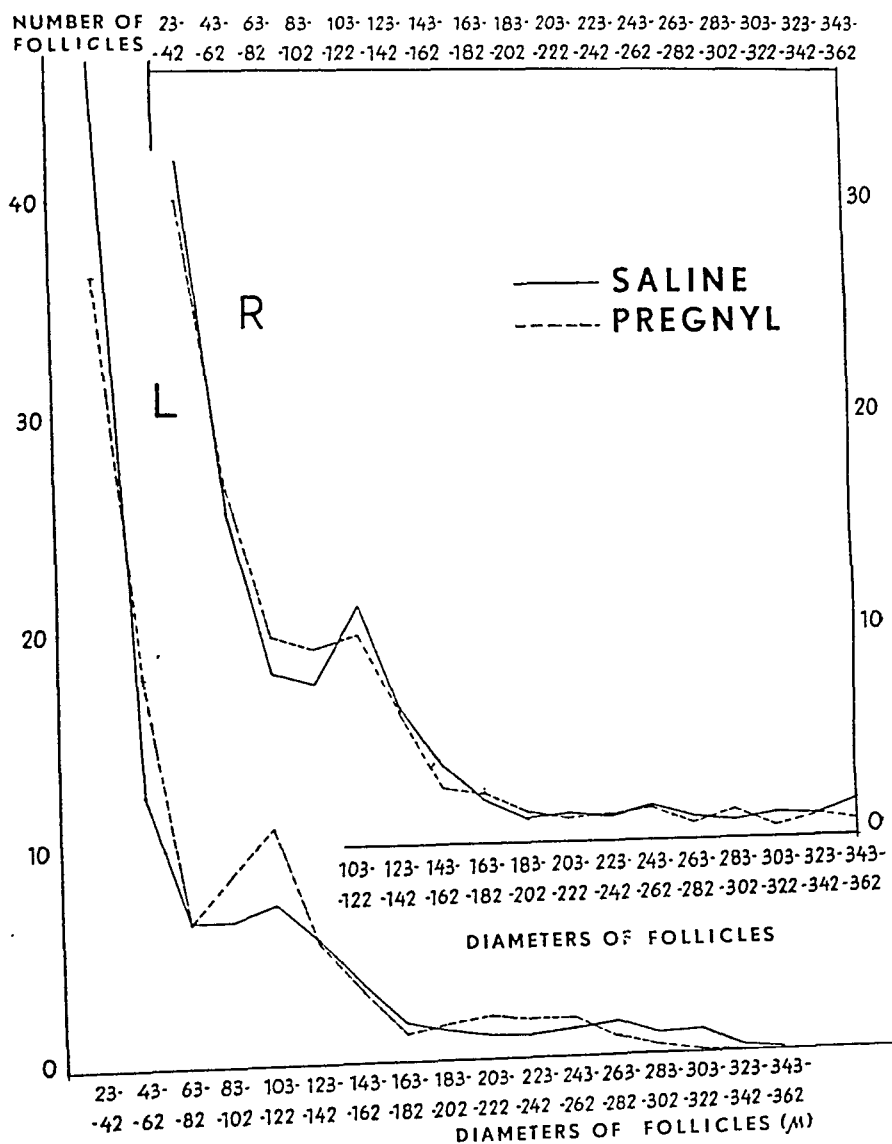


Fig. 2.

Number of follicles in each size-group as a percentage of the total number. In each column two size-groups from Fig. 1 have been combined.

follicles down (v. infra, sub. B). As soon as treatment was stopped, however, they were able to increase in number, i. e., between the 8th and the 17th day.

- B. The average increase in the number of the smallest follicles amounted in B to 38 per cent only (av. of 24, 43, 44 and 45 per cent), whereas in A, it was 71 per cent. As such a difference was also found when Pregnyl was administered between the 8th and the 17th day after hypophysectomy, it is likely that chorionic gonadotrophin inhibited the increase in the number of primordial follicles after hypophysectomy.

*The largest follicles.*

- A. Follicles with a diameter of more than 330  $\mu$  disappear almost completely within 7 days. Before hypophysectomy, a diameter up to 576  $\mu$  was reached, whereas one week later the largest diameter was 360  $\mu$  (cf. Fig. 1). Even in these large follicles we found a large number of mitoses on the 17th day after hypophysectomy. *Swezy* (1933) also noted this and we must therefore assume that the granulosa continues its growth after removal of the hypophysis.
- B. In the Pregnyl-group the follicles larger than 290  $\mu$  had disappeared. Although the difference as compared with A is not large the data for each rat suggest its significance (Table 1).

Table 1.

Large follicles in each animal, at hypophysectomy and 7 days later.

Rat Nr.		Number of follicles					
		Larger than 322 $\mu$		292—332 $\mu$		252—292 $\mu$	
A. No. treatm.	B. Chor. gonad.	A. No. treatm.	B. Chor. gonadotr.	A. No. treatm.	B. Chor. gonadotr.	A. No. treatm.	B. Chor. gonadotr.
		r. ov. l. ov.	r. ov. l. ov.	r. ov. l. ov.	r. ov. l. ov.	r. ov. l. ov.	r. ov. l. ov.
4583	4574	5—0	24—0	7—10	10—0	3—16	4—0
4813	4575	19—0	8—0	9—2	4—1	6—4	5—14
4814	4576	22—0	16—0	5—0	3—0	10—2	1—5
4815	4809	28—2	14—0	4—9	8—0	8—16	5—3
Results:		disapp.	disapp.	n. disapp.	disapp.	not disapp.	not disapp.

Fig. 2 also shows this »shifting back« of the largest follicles to lower size-groups in the Pregnyl-ovaries (dotted line), as compared with the untreated hypophysectomized animals. We refer to this later.

### *Atresia.*

In Fig. 1 the part of each column above the transverse dividing line indicates the number of follicles in the initial phase of atresia.

- A. The percentage of subatretic follicles is the same 7 days after hypophysectomy as it was at the time of the operation (16 resp. 17 per cent). This suggests that the decrease found after hypophysectomy in the number of follicles with a diameter of more than  $32\ \mu$  is not caused by an increased »mortality« of the follicles, but by an insufficient replacement, i. e., the number of primordial follicles that develop into follicles of medium size per unit of time undergoes a decrease. Hypophysectomy appears to have no marked influence on the »life-time« of the follicle.

The highest percentage of subatretic follicles is found among the smallest and largest groups (Fig. 3). Hypophysectomy does not cause a marked change in the general trend of this curve.

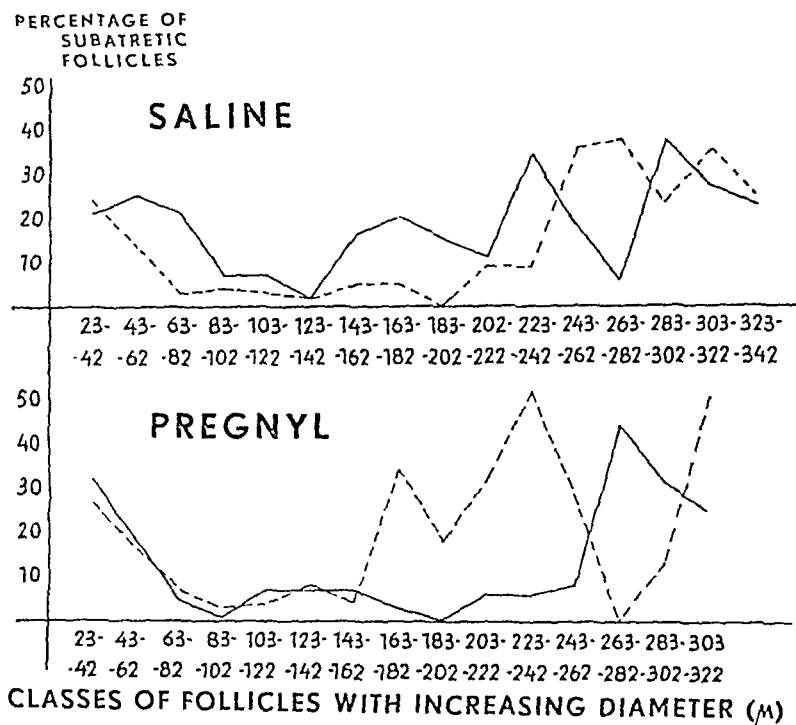
- B. Here too the percentage of atretic follicles proved to be unchanged (17 as compared with 18 per cent). Chorionic gonadotrophin may cause increased atresia in normal animals (*Herlant*, 1931; *Selye et al.*, 1933; *Desaive*, 1935; *Guyénot et al.*, 1935; etc.). It certainly does not do so, however, in hypophysectomized rats.

Fig. 3 reveals a difference between A and B in the *distribution* of atresia in the different size-groups. In B the enhanced atresia of the larger follicles begins at  $143\ \mu$ , in A at  $223\ \mu$ . In A the percentage of subatretic follicles of  $143$ — $222\ \mu$  decreased in all four rats, whereas in three of the four Pregnyl-treated animals there was a considerable increase.

*Follicles of intermediate size (33—312  $\mu$ ).*

For the sake of convenience these follicles are dealt with en bloc.

The number of follicles belonging to these size-groups decreased to 86 per cent (av. of 93, 77, 78 and 96 per cent).



*Fig. 3.*

Percentage of subatretic follicles in each size-group.

Drawn line: before treatment (right ovaries).

Dotted line: after treatment (left ovaries).

In the Pregnyl-group, the number of follicles of 33—312  $\mu$  decreased to 91 per cent (av. of 75, 92, 96 and 102 per cent). It appears as if a number of follicles which otherwise would have attained much larger diameters now became atretic at a smaller size and measured between 183 and 262  $\mu$ : since (1) in all size-groups between 183 and 262  $\mu$  the number of follicles — as a percentage of their total number — is higher in B than in A, whereas in

all higher size-groups the reverse is true (Fig. 2 L), (2) the largest diameter attained by the follicles is decreased and (3) Pregnyl causes a shifting back of the increased atresia of the largest follicles to those of a smaller size. As, however, (considering the unchanged total percentage of atretical follicles), their »lifetime« does not seem to have been shortened by chorionic gonadotrophin, a slowing down of their growth-rate, at least from 183  $\mu$  upwards, must be assumed.

Probably the development of smaller follicles has also been impaired by Pregnyl, as shown by the accumulation of follicles measuring ca. 100  $\mu$ . (Fig. 2 L).

#### *Cavity-formation.*

In the majority of the follicles cavity-formation begins when a diameter between 93 and 192  $\mu$  is reached.

- A. Before hypophysectomy there were 391 follicles measuring between 93 and 192  $\mu$ , of which 47 per cent contained cavities. After 7 days there were 250 of them, with 52 per cent cavities. Thus, the number of follicles, belonging to these size-groups, was markedly decreased, as was seen before (Fig. 1) but the percentage of cavity-containing follicles apparently did not change.
- B. In the Pregnyl-group these figures are: before treatment 312 follicles measuring 93—192  $\mu$ , with 50 per cent cavities, after treatment 291 follicles, with 57 per cent cavities. Pregnyl has thus checked the decrease in number occurring after hypophysectomy. This has already been stated in the preceding section. The percentage of cavity-containing follicles did not change conspicuously. This leads us to believe that in addition to follicle-*growth*, follicle-*maturation* was affected by chorionic gonadotrophin.

### DISCUSSION

The increase in number of the smallest follicles following hypophysectomy might be explained by assuming that under normal condition the hypophysis exerts an inhibitory effect

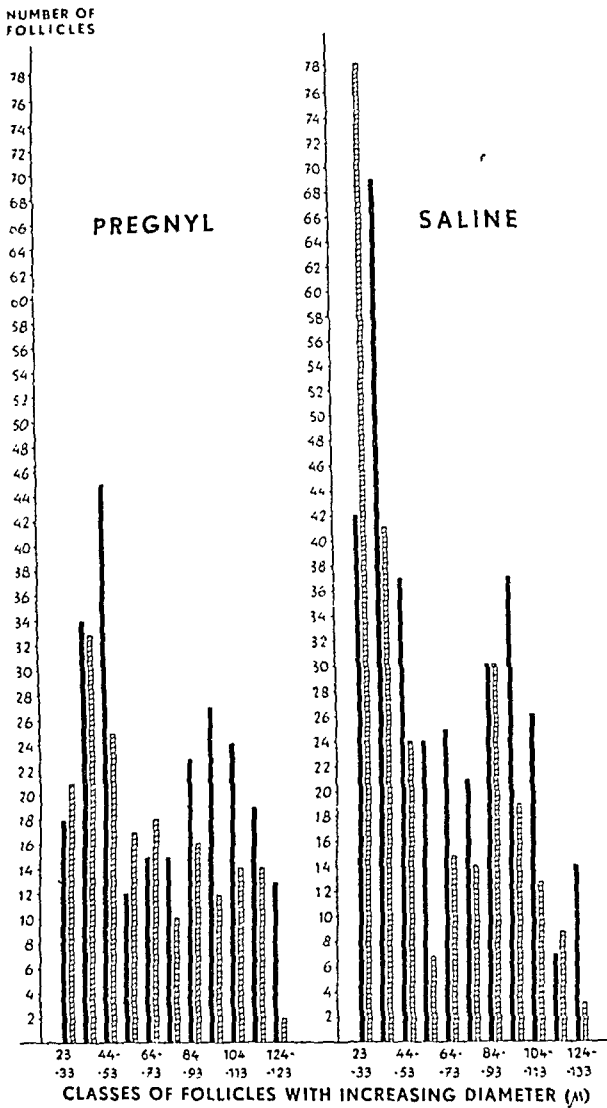


Fig. 4.

Number of follicles in the ovaries of young hypophysectomized rats. Treatment: Pregnyl (5 I. U. daily) during 7 days following hypophysectomy; removal of right ovary on the 8th day; further treatment during 10 days with Pregnyl (5 I. U. daily, 5 animals) or saline (4 animals); removal of left ovary.

Black columns: numbers in right ovaries.

Shaded columns: numbers in left ovaries.

on the development of the ova to primordial follicles. The increase in number would thus be due to the elimination of this inhibition. Arguments against this interpretation are:

(a) Like larger follicles, the primordial follicles were found to increase in number when an adequate dose of pituitary gonadotrophic hormone was supplied (see next paper) and

(b) There was no increase in the *total* number of follicles in any of our hypophysectomized rats.

Thus we are inclined to ascribe the accumulation of the primordial follicles after hypophysectomy entirely to a decrease in the number of these follicles which develop into larger follicles per unit of time. By this we do not imply that the preceding development of primordial ova into primordial follicles proceeds at the same rate as before hypophysectomy. It may even be impaired, but in that case further growth to medium-sized and large follicles must have been far more seriously affected if the accumulation of small primordial follicles is to be explained.

Our results are in good agreement with those of *Swezy*, who observed an increase in number in her lowest size-group a few weeks after hypophysectomy. This size-group, however, contained both primordial follicles and primordial ova. Since the number of primordial follicles might not exceed a few hundred, whereas *Swezy's* joint number of ova and primordial follicles increased by as many as *thousand*, her figures put the increase in the number of *ova* beyond doubt. Our observations furnish evidence that small primordial follicles are also involved in this increase.

Does the production of new ova and the formation of the first layer of granulosa cells take place to a certain extent without hormone stimulus? Several authors have shown that hormone-release occurs if the hypophysis is touched e. g. during hypophysectomy (*Dubowik*, 1930; *Lane & Greep*, 1935; *Williams*, 1945). However, these observations mainly relate to medium-sized and large follicles, which in our experiments did not increase in number. Apart from such a release of hormone there might be stores of pituitary gonadotrophin, e. g.

in the ovary. In both cases we must assume that the primordial ova are more sensitive to these small amounts of hormone than are the follicles. However, it would then be difficult to explain the negative results of Pregnyl administration in our experiment, as the well-known enhancement of the effect of pituitary hormone by chorionic gonadotrophin applies to ova as well as to follicles (see next paper). Moreover, the primordial follicles still increased in number after the 8th day following hypophysectomy, when it is highly unlikely that stores of pituitary gonadotrophin were still present.

It is conceivable that stored *oestrogen* caused the growth of ova. Formation of new oocytes or proliferation of the germinal epithelium in the oestrous-stage of the cycle has been described in several species of animals (*Evans & Swezy*, 1931; *Allen & Creadick*, 1937; *Bullough*, 1942; *Schmidt & Hoffmann*, 1941; *Everett*, 1942). However, we should then be compelled to assume a special marked sensitivity of the ova to oestrogen, whereas the primordial follicles would be less sensitive. We are as yet not inclined to this theory, as oestrogen favours to a marked degree the development of smaller follicles to those of a medium size in hypophysectomized animals (*Pencharz*, 1940; *Simpson et al.*, 1941; *Gaarenstroom*, 1942; *Gaarenstroom & de Jongh*, 1943; *Williams*, 1944).

Four months after transection of the hypophyseal stalk *Blair Bell* (1919) found degenerated primordial ova only. *In the long run* the presence of a functioning hypophysis therefore might be indispensable for the existence and growth of ova.

The best interpretation of the available data seems to be offered by assuming that follicle growth becomes increasingly dependent on the hypophysis during the course of its development. Thus, impairment by hypophysectomy becomes apparent earlier in the larger size-groups, whereas in the lower it will only be easily perceived after a long time lag.

The development of the follicle from the primordial stage onward was clearly affected in the first week following hypo-



physectomy. As we found, rather unexpectedly, that the percentage of subatretic follicles was unchanged, it seems unlikely that the »life-time« of the follicles had been shortened. Thus, the decrease from 576 to 360  $\mu$  of the largest diameter attained by the follicles might be due to a slowing down of their growth rate. Evidently, this slowing down did not affect all follicles to the same extent, since a whole series of size-groups (173—322  $\mu$ ) did not show any change (Fig. 1 A).

In the hypophysectomized rat, chorionic gonadotrophin seems to impair follicles growth at the beginning and towards the end: it causes the number of primordial follicles to be decreased and probably affects the development of at least those follicles measuring more than 180  $\mu$ . Evidence for the last-named effect has been put under different headings in the text. The first action may be due to: (a) Increased atresia of primordial follicles. Such an increase, however, was not found to have occurred, (b) Stimulation of follicle-growth, resulting in »exhaustion« of the stock of primordial follicles. This cannot be accepted as an explanation, however, as chorionic gonadotrophin caused a reduction in the number of medium-sized and large follicles. So we are inclined to assume: (c) *a reduction in the number of ova which develop into primary follicles, per unit of time.*

This is in good agreement with observations made by Gaarenstroom & de Jongh (1946, p. 119), who were able to suppress the onset of follicle-growth in 10-day-old rats by the administration of chorionic gonadotrophin or of testosterone. We have demonstrated that chorionic gonadotrophin induces the production of an androgenic substance in the ovary of hypophysectomized young rats (Paesi & Gaarenstroom, 1943; Gaarenstroom & de Jongh, 1946, p. 92) and thus, the observed effects of Pregnyl may have been due to the intervention of an androgen. We hope to confirm this view in subsequent studies.

## SUMMARY

Hypophysectomy causes an accumulation of small primordial follicles and a decrease in the number of follicles belonging to higher size-groups. Evidently, the development of the smallest primordial follicles into larger follicles is more markedly impaired by the removal of the hypophysis than the preceding development of the primordial ova into primordial follicles.

The largest diameter reached by the ovarian follicle in the hypophysectomized animal is subnormal. This is probably due to a slowing-down of the growth rate of the follicles. Since the percentage of atretic follicles did not change, it is unlikely that their average »life-time« was seriously affected.

Chorionic gonadotrophin produces in the hypophysectomized rat a decrease in the number of ova which develop into primordial follicles per unit of time and probably causes a reduction in the rate of growth during the last stages of follicular development. The latter conclusion is based on data derived from the distribution of atresia over various size-groups, the unchanged percentage of atretic follicles and changes in the numbers of medium-sized and largest follicles. Contrary to evidence obtained in normal animals, chorionic gonadotrophin did not increase atresia in hypophysectomized rats.

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## THE SENSITIVITY OF THE MAMMARY GLAND TO OESTRONE IN DIFFERENT STRAINS OF MICE WITH AND WITHOUT MAMMARY TUMOUR AGENT

BY

O. MÜHLBOCK

The development of cancer of the breast in mice is dependent on four factors: 1. genetic constitution; 2. an agent, »mammary tumour agent«, probably a virus which is transferred through the milk of the mother to the young (»milk factor«); 3. hormonal influences, mainly of the ovarian type and 4. influences of environment. Whether or not the last-mentioned factors have a direct influence or act only through the influence of the hormones is uncertain.\*)

For a long time particular attention has been given to the oestrogens. No doubt the oestrogenic hormones play an important role in the development of mammary cancer in mice. In order to obtain further insight into the influence of this hormone, the oestrous cycle in females of high cancer strains has been regularly controlled and compared with that of low-cancer strains. (Korteweg, 1933, Lacassagne, 1934, Bonser, 1935, Brunschwig and Bissel, 1936 and Deringer *et al.*, 1945). Differences were found between the high-cancer and low-

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\*) A survey is found in: *A symposium on mammary tumors in mice by members of the staff of the National Cancer Institute*; Am. Ass. Adv. Sci., 1945.

cancer strains which were not necessarily connected with the development of mammary cancer. This opinion was strengthened by investigations of strains which, by appropriate foster-nursing, did or did not possess the mammary tumour-agent («milk factor») (*Armstrong, 1948, Huseby & Bittner, 1947 and Bittner, 1948*). Although the presence or absence of the mammary tumour agent drastically changed the frequency of mammary cancer, it was found that this agent exerted no influence on the course and frequency of the oestrous cycle. In a second series of experiments the amount of oestrogen necessary to bring about oestrus in castrated females of different high-cancer and low-cancer strains was determined. (*Korteweg, 1935, Van Gulik & Korteweg, 1940, and Mühlbock, 1947*). Here also differences were found between the several strains which are not related to the presence or absence of the mammary tumour agent. (*Shimkin & Ander-vont, 1941*).

In a previous publication (*Mühlbock, 1948*) the activity of the oestrogenic hormone was determined not on the vagina but on the mammary gland, as this is the site of origin of the cancer. The strains examined showed that the mammary gland of high-cancer and low-cancer strains was equally sensitive to oestrone, while the vagina in the low-cancer strain was more sensitive than in the high-cancer strain. It was thought that the relatively greater sensitivity to oestrone of the mammary gland of high-cancer strains could be attributed to the mammary tumour agent. To test this supposition experimentally, the sensitivity of the mammary gland to oestrone\*) was determined in three different pure strains with and without a mammary tumour agent.

## METHOD

Mice of the following pure strains were examined (for the origin of strains see: »A symposium on mammary tumors in mice«).

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\*) Oestrone was supplied by Dr. M. Manus, N. V. Organon, Oss. Holland.

- 1st. »d« Dilute-brown, (Murray-Little), high-cancer-strain with mammary tumour agent and with a high percentage of mammary cancer in virgins and breeders.
- 2nd. »dz« Dilute-brown, without mammary tumour agent. Just before birth the young were removed from the uterus by operation and foster-nursed by females of the C 57 Black strain. This procedure prevents the development of mammary cancer in these animals except in a few cases.
- 3rd. »A« (Strong). Cancer strain with mammary tumour agent and with a low percentage of mammary cancer in virgins and a high percentage in breeders.
- 4th. »Az«. A-strain without mammary tumour agent as in »dz« foster-nursed by females of the C 57 black strain.
- 5th. »B«. C 57 Black (Little). Non-cancer strain without mammary tumour agent.
- 6th. C 57 Black, foster-nursed immediately after birth by ♀ d (cancer strain); with mammary tumour agent. After foster-nursing the frequency of mammary cancer is small. All these foster-nursed animals have, as *Korteweg* (1948) has shown, the »hyperplastic nodules« in

Table 1.

Growth of the mammary gland in castrated mice after administration of oestrone.

+ = growth; — = no growth.

Oestrone dose in $\gamma$	♀ d with agent	♀ dz without agent	♂ d with agent	♂ dz without agent	♀ A with agent	♀ Az without agent	♀ B without agent	♂ B with agent
0,01					—	—	—	
0,05					+	+	(+)	(+)
0,1	—	—	—	—	+	+	+	+
0,2	(+)	(+)	(+)	(—)	+	+	+	+
0,3	+	+	+	+				

the breast which is typical of the presence of the mammary tumour agent.

All animals were reared in the Institute and fed with commercial pellets and tap-water ad libitum.

In the »d« and »dz« strain, both females and males were examined, but in the other strains only the females. The animals were castrated when they were 3—4 weeks old. The animals of the A- and of the B-strain were examined 2—3 weeks after castration and those of the d-strain 2—3 months after castration.

The sensitivity of the mammary gland to oestrone was determined according to the method of *Lewis & Turner* (1942), as previously published (*Mühlbock*, 1948 a, b).

## DISCUSSION OF THE RESULTS

The summary of results is tabulated (Table 1). In all three strains examined the sensitivity of the breast to oestrone is independent of the presence of the mammary tumour agent. In the A- and B-strain, examined shortly after castration, the liminal dose of oestrone lies between 0.05  $\gamma$  and was examined three months after castration. The oestrone-0.1  $\gamma$ ; in the d-strain the dose is 0.3  $\gamma$ , because the strain dose is the same in both males and females.

*Bonser* (1945), in an investigation on the development of cancer of the breast in mice, considered the possibility that the oestrogenic hormone »would appear to act as a developing factor upon a breast already sensitised by the milk factor«. This explanation however does not agree with the results of the present investigation. Only a very limited part of the growing phase of the breast is controlled in this way; it is possible, however, that a synergistic activity of the milk-factor with the action of oestrone can be observed only at a later phase of the growth process, e. g., only when, under the influence of hormones, the stage of hyperplasia has been reached.

It was stated in the introduction that the mammary tumour

agent exerts no effect either on the course of the oestrous cycle, or on the dose of oestrone necessary to induce oestrus in castrated animals. The correlation between the oestrous cycle and the oestrogenic dose of oestrone suggests that the production of oestrogen is not influenced by the mammary tumour agent.

There is another possible influence on the ovarian hormone production, i. e., an influence of the mammary tumour agent on the initiation or duration of production of the hormone. The period during which the ovarian hormone is produced is, however, difficult to determine. In animals with mammary tumour agent, the development of a breast cancer before senility makes observation impossible. The irregularity of the oestrous cycle often found in older animals also makes judgment difficult. *Armstrong* (1948) was not able to determine any influence of the mammary tumour agent on the beginning of the menopause. The beginning of a full hormone production can be characterized by the first opening of the vagina, the first oestrus or the first pregnancy. The opening of the vagina in different genetic strains was found by *Deringer et al.* (1945) to commence at different ages. In the strains which had a higher tumour-frequency the vagina opened earlier. Recently, *Armstrong* (1948) found that in pure strains no influence was exerted by the presence or absence of the mammary tumour agent. The time at which animals, mated with their brothers.

Table 2.  
Time of the first pregnancy.

Strain	Number	With or without mammary tumour agent	Mean-age in days of first delivery
d	122	with	89
dz	86	without	86
A	112	with	77
Az	23	without	79
B	51	without	87
B foster-nursed by ♀ dba.	34	with	80



made their first nest, was compared in the different strains examined.

It is evident from Table 2 that the time of the first pregnancy is also independent of the mammary tumour agent.

(The assistance of Miss W. van Ebbenhorst Tengbergen is gratefully acknowledged.)

### SUMMARY

In three genetically pure strains of mice (d; A; B) which by appropriate foster-nursing did or did not possess the mammary tumour agent the sensitivity of the mammary-gland to oestrone was determined. The development of the mammary-gland following the application of oestrone is not influenced by the mammary tumour agent.

The influence of the mammary tumour agent on ovarian hormone production and hormonal action is discussed. There is at present no evidence that the mammary tumour agent influences the production or activity of the oestrogenic hormones.

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## THE EFFECT OF OESTROGEN ON THE PHOSPHATE TURNOVER IN THE HYPOPHYSEAL- DIENCEPHALIC SYSTEM

BY

U. BORELL and A. WESTMAN

The question whether or not oestrogen has a stimulating effect upon the functional activity of the ovaries has been much discussed. Some authors believe that oestrogen therapy acts in the same way as a substitution treatment. Others, again, express the view that under certain conditions it may be regarded as a stimulation therapy. The studies of the effect of oestrogen treatment in secondary amenorrhea made by *Kaufmann* (1936, 1938), and by *Westman* (1941) lent support to this assumption. The latter study has shown that a normal menstrual cycle was reestablished following oestrogen therapy in almost one half of the cases. *Rydberg & Mathiesen* (1948) published a paper supporting the view of a stimulating effect of oestrogen on ovarian functional activity. They found that the level of oestrogenic substance in the urine, very low prior to treatment, increased considerably in some cases following the administration of Sexadien [Di- (p-oxyphenyl)- hexadien] which is rapidly excreted. This observation was explained by assuming that this synthetically prepared oestrogenic substance has a stimulating effect upon the pituitary gland, and that this effect is maintained and is followed by a return of the functional activity of the ovaries.

Hence, it is justifiable to assume that oestrogen therapy

may occasionally combine the properties of both substitution and stimulating therapy. At the present state of our knowledge it is not possible to explain the variability in the effect of oestrogen. Although many authors have given special attention to this problem, the results hitherto obtained have been either inconsistent or difficult to interpret.

In this connection the observations made by *Jones & Mac Gregor* (1936) on women at the menopause are interesting. They reported that following oestrogen therapy the level of gonadotrophic hormone in the urine decreased considerably. They came to the conclusion that oestrogen inhibits the production of gonadotrophic hormone.

The results of experiments on animals have also been inconsistent. *Meyer, Leonard, Hisaw & Martin* (1932) reported that in rats the amount of gonadotrophic hormone decreases following oestrogen administration. *Hohlweg* (1935) found that oestrogenic hormone induces corpus luteum formation in the ovaries of juvenile rats. This observation strongly suggests that oestrogen exerts a stimulating action on the pituitary gland.

It is not within the scope of this paper to review the abundant literature concerned with the problems discussed here. The experiments to be reported were performed in order to determine whether oestrogens have a stimulating effect upon the functional activity of the ovaries. From the therapeutic point of view it is of prime importance that this problem should be clarified.

In this study we have investigated the effect of oestradiol monobenzoate on the functional activity of the hypophyseal-diencephalic system.

As described in preceding papers (*Borell, Westman & Örström*, 1947, 1948), we investigated the functions of this system by means of radioactive phosphate. We found that in rabbits, decapitated two minutes after coitus, the phosphate turnover in the tuber cinereum and the adenohypophysis was increased while the phosphate turnover in the ovaries did not increase until thirty minutes after coitus.

An increase in the phosphate turnover in the pituitary gland was also observed in castrated rabbits. This corresponds to a great extent with the increase in the production of gonadotrophic hormone found characteristically in the pituitary gland of castrated animals.

Modification of the phosphate turnover in the tuber cinereum and the adenohipophysis during the different phases of the normal sexual cycle were also observed in rats.

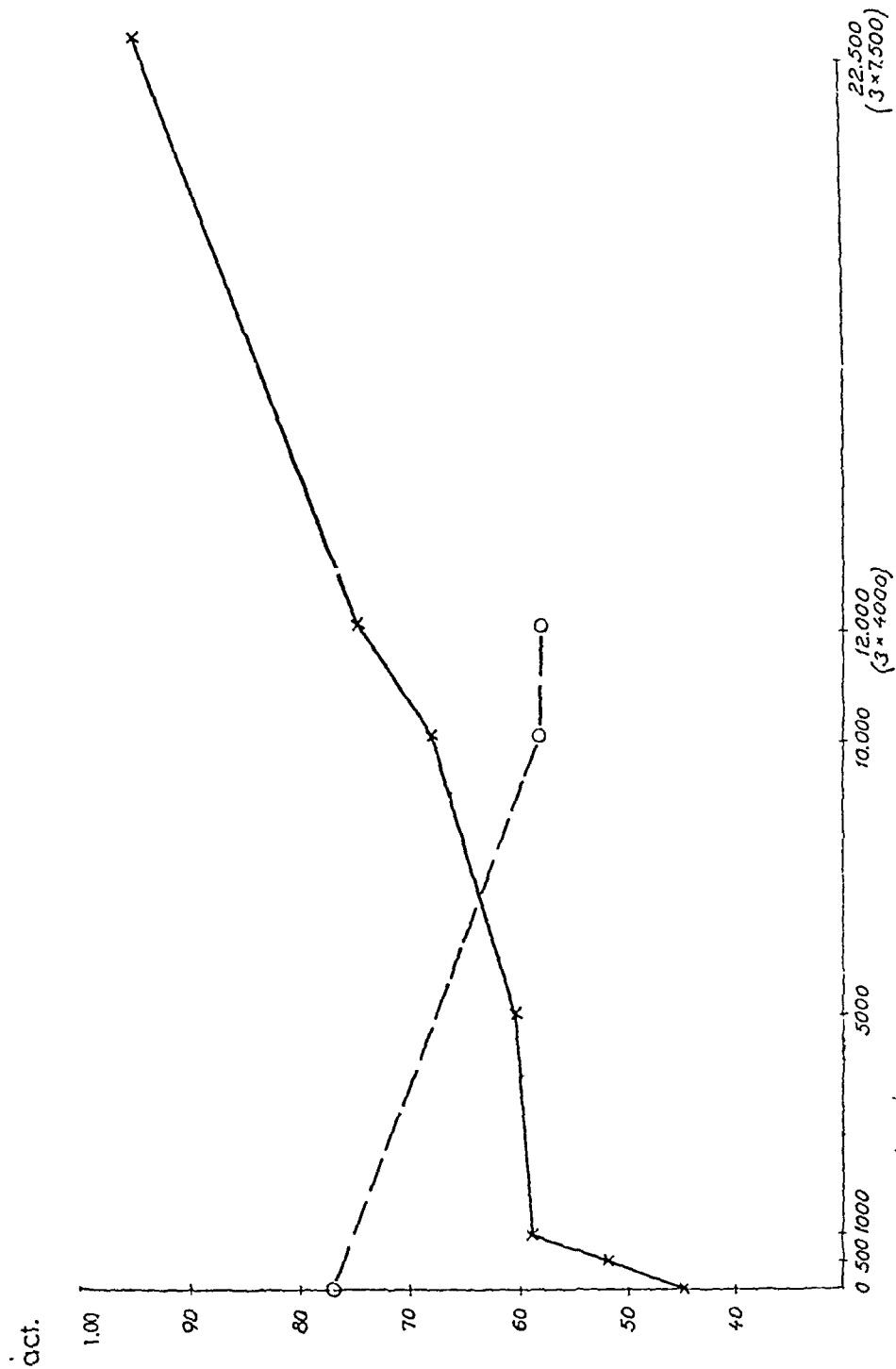
### MATERIAL AND METHOD OF STUDY

The experiments were performed on 105 white rats weighing from 80 to 145 gm. Twenty-four of them were hypophysectomized. After this procedure, the animals were controlled by daily study of the vaginal smears for at least three weeks. Hypophysectomy was controlled by histological investigation of the sella.

All animals were treated with oestradiol monobenzoate, administered by intramuscular injections.

The non-hypophysectomized animals were divided in two groups. In one group single doses of 500, 1000, 5000 and 10000 I. B. U., respectively, were injected, and the animals were decapitated forty-eight hours after the injection. In the other group, either 4000 or 7500 I. B. U. were injected daily over three days, and the animals were killed forty-eight hours after the last injection.

In boths groups 0.018 mC  $^{32}\text{P}$  in 5 per cent glucose solution were injected intraperitoneally forty minutes before decapitation. At autopsy, the radioactivity and the total phosphate of the blood, cerebellum, tuber cinereum, adenohipophysis and ovaries were determined. As the tuber cinereum and the adenohipophysis of a single animal were found to be too small for determining the radioactivity and total phosphate, the organs of three animals were put together and analysed as a unit. The values obtained are expressed in terms of specific activity, i. e. in impulses per  $\gamma$  P.



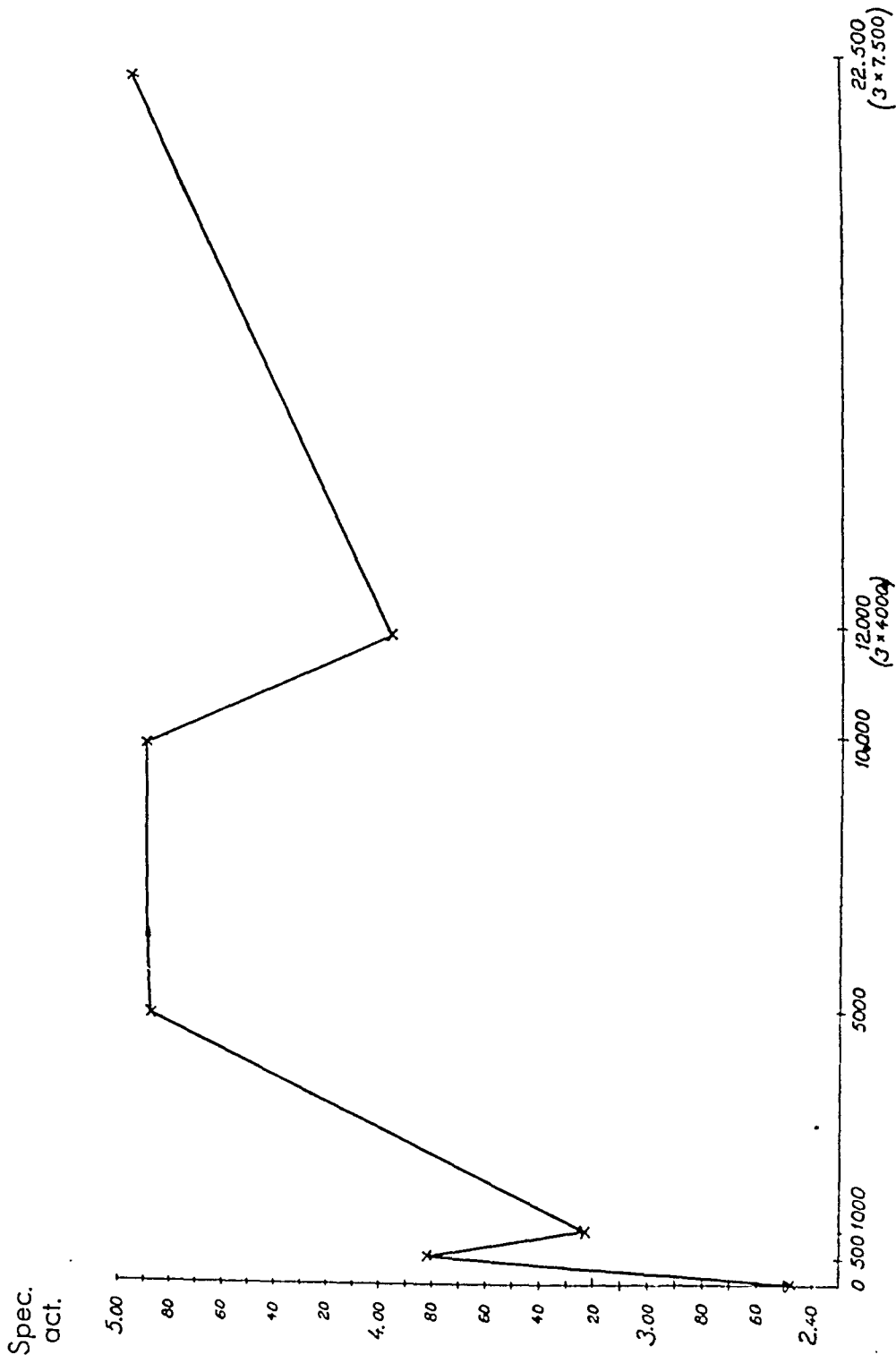


Fig. 2.

Effect of oestradiol monobenzoate on the  $^{32}\text{P}$  — turnover in the adenohypophysis.  
 Ordinates: Specific activity. Abscissae: Dose of oestradiol monobenzoate in I.B.U.

## RESULTS AND DISCUSSION

Table 1 shows the changes in the specific activity following injection of oestradiol monobenzoate.

Table 1.

Specific Activity in the Tuber Cinereum, Adenohypophysis, Ovaries, Whole Blood and Cerebellum following Injection of Different Amounts of Oestradiol monobenzoate in Normal Rats.

Amount injected in I. B. U.	Tuber Cinereum	Adeno-hypo-physis	Ovaries	Whole Blood	Cerebellum
0	0.45	2.48	8.0	25.0	0.22
500	0.52	3.81	10.4	20.3	0.26
1000	0.59	3.23	10.1	26.4	0.26
5000	0.61	4.88	11.7	23.1	0.30
10000	0.69	4.92	9.6	17.6	0.29
3 × 4000	0.76	3.99	8.5	26.4	0.24
3 × 7500	0.97	5.00	9.8	18.4	0.28

*Tuber cinereum.* It required a single dose of 10000 I. B. U. to produce an appreciable increase in the phosphate metabolism as compared with the control values. The increase was more marked with frequent injections of large doses. (Fig. 1).

The observation that radioactivity increases in the tuber cinereum after injection of large amounts of oestrogen raised the question whether oestrogen exerts a direct action on the diencephalon or an indirect action via the pituitary gland. To clarify this hypophysectomized rats were used as test objects.

As Table 2 shows there was no significant change in the radioactivity of the tuber cinereum even after large doses of oestradiol monobenzoate.

*Adenohypophysis.* Oestradiol monobenzoate produced an increase of radioactivity. The increase was clearly noticeable even after the administration of 5000 I. B. U. In normal animals the phosphate turnover in the adenohypophysis is about five times as high as in the diencephalon. Expressed as a per-

centage, however, the increase of the phosphate metabolism produced by oestrogen seems to be the same in these two organs (Fig. 2).

Table 2.

Specific Activity in the Tuber Cinereum, Ovaries, Whole Blood and Cerebellum following Injection of Different Amounts of Oestradiol monobenzoate in Hypophysectomized Rats.

Amount injected in I. B. U.	Tuber cinereum	Ovaries	Whole Blood	Cerebellum
0	0.77	16.6	52.7	0.50
10000	0.59	10.8	46.0	0.29
$3 \times 4000$	0.59	24.2	51.8	0.42

*Ovaries.* There was no evidence of increased radioactivity after the injections; at any rate, it was not measureable with our present method of assay. The explanation of this finding must probably be sought in the comparatively short period of time during which the experiments were made.

*Blood.* The values vary considerably. Constantly occurring changes attributable to the action of oestrogen were not observed.

*Cerebellum.* Contrary to the observations made on blood, the specific activity in the cerebellum are just the same in the different groups.

The specific activity in the blood and cerebellum was determined primarily to obtain control values. Changes comparable to those observed in the tuber cinereum and adenohypophysis were not found in the blood or in the cerebellum.

Our experiments have shown that oestradiol monobenzoate administered in comparatively large doses produces an increase in the phosphate turnover in the adenohypophysis. This observation supports the assumption that oestrogen administered in amounts equal to those used in this study has a stimulating effect on the adenohypophysis.



Recently there has been an increasing tendency to consider the hypophyseal and diencephalic systems as a functional entity. This led us to determine the  $^{32}\text{P}$  turnover in the tuber cinereum. We found that its increase is dependent on the amount of oestrogen injected. As these changes were not observed in hypophysectomized rats it is possible that oestrogen first influences the hypophysis and secondarily produces modifications in the phosphate turnover of the tuber cinereum.

### SUMMARY

In normal rats oestradiol monobenzoate in comparatively high doses increased the  $^{32}\text{P}$  turnover in the adeno-hypophysis and tuber cinereum. In hypophysectomized rats it had no significant effect in the tuber cinereum.

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## TESTOSTERONE TREATMENT AND 17-KETOSTEROID EXCRETION

### II. ADMINISTRATION OF TESTOSTERONE PROPIONATE. EMULSIFIED IN WATER

BY

CHRISTIAN HAMBURGER

Quite recently *Lens, Overbeek & Polderman* (1949) introduced a new method of dispensing steroid hormones. The crystalline steroids are dissolved in benzyl alcohol and from this solution emulsions are prepared. When the emulsions are diluted with an equal volume or more of water or serum the benzyl alcohol dissolves in the water resulting in the formation of a crystalline precipitation.

This method of dispensing steroid hormones was thought to possess several advantages over those commonly used, viz. oily solutions and suspensions of crystals. Thus, very thin needles can be used for the intramuscular injections of the emulsions, and the absorption from the crystals precipitated in the tissues should presumably be much slower than from oily solutions. The authors (l.c.) described the technique used for the preparation of emulsions of testosterone propionate and oestradiol monobenzoate and reported the results of some animal experiments. A comparison was made of the growth of the capons' comb after one intramuscular injection of testosterone propionate in oily solution, in emulsion and in crystal suspension. The influence of the mode of administra-

tion of oestradiol monobenzoate and of other oestrogenic substances was determined by means of the vaginal response of adult spayed rats. In these animal experiments, the effects produced by the emulsions were found to be comparable to or even better than those of crystal suspensions. No pain and local or general reactions were noted in six men, after intramuscular injection of 1 ml. of the emulsions; apart from this, the clinical effects of the emulsified preparations have not been examined.

*Hamburger & Kaae* (1949) have shown that an objective evaluation of the absorption rate of testosterone propionate after various modes of administration can be obtained by determinations of the daily 17-ketosteroid excretion in the urine. After a single intramuscular injection of testosterone propionate in oily solution an increased 17-ketosteroid excretion was found for 3 to 5 days, and for an average of 12 days after the injection of a crystal suspension of the same substance. Furthermore, the maximal excretion occurred in the first 24-hour urine after the injection of oily solutions, but on the 3rd to 5th day after the injection of crystal suspensions. It was, therefore, reasonable to assume that important information might be gained from determinations of the 17-ketosteroid excretion in man after the injection of emulsions of testosterone propionate.

By the courtesy of Dr. *Frederik Paulsen*, »Nordiska Organon«, Stockholm, we were supplied with a number of ampoules of »Neo-Hombreol«, each containing 50 mg. testosterone propionate in aqueous emulsion.

## MATERIAL AND METHODS

Alcoholic dilutions of one of the ampoules were assayed by the comb growth method (direct application technique), the activity being compared with that of a known preparation of crystalline testosterone propionate. The activities agreed within  $\pm 20$  per cent.

*The experimental subjects* were four healthy men, 31 to

47 years old. The preparations (emulsions and oily solutions) were given as intramuscular injections. Determinations of the 24-hour urinary 17-ketosteroid excretion were carried out for some days before the injection, on the day of injection, and for some time afterwards. The urines were extracted and assayed by the »micro-ether-method« (*Hamburger & Rasch*, 1948), and the calculations of the amount of testosterone propionate excreted as 17-ketosteroids were carried out as described by *Hamburger & Kaae* (1949).

## RESULTS

*Expt. No. 1* (Fig. 1, A) was carried out by the author on himself. The average daily excretion of 17-KS was 13.7 mg. for a period of 10 days before the injection. Three millilitres of »Neo-Hombreol« emulsion were injected intramuscularly in the regions of the hip ( $1.0 + 2.0$  ml.). In the course of the first 24 hours 69.0 mg. 17-KS were excreted, and the values for the following days were 27.4, 17.7 and 13.8 mg., respectively. The maximal excretion occurred on the first day, and the increased excretion lasted for 3 (or 4) days. The »extra excretion« amounted to 73.1 mg., from which figure it was calculated that 58 per cent of the 150 mg. TP injected was excreted as 17-KS. The low 17-KS excretion on the fifth day, and the gradual rise to pre-treatment values, indicate an inhibition of the hypophyseal functions. Assuming that the inhibition had been present from the day of injection, 67 per cent of the TP would have been excreted as 17-KS. The true value must therefore be found somewhere between 58 and 67 per cent. The excretion pattern closely resembled that previously observed after the injection of 150 mg. TP in oily solution to the same experimental subject (see; *Hamburger & Kaae*, 1949, Fig. 4). Some hours after the injection of the emulsion, local pain and tenderness were observed, and the body temperature was moderately raised in the evening. The tenderness disappeared in the course of the next two days.

*Expt. No. 2* (Fig. 1, B). In this case 100 mg. of »Neo-Hom-

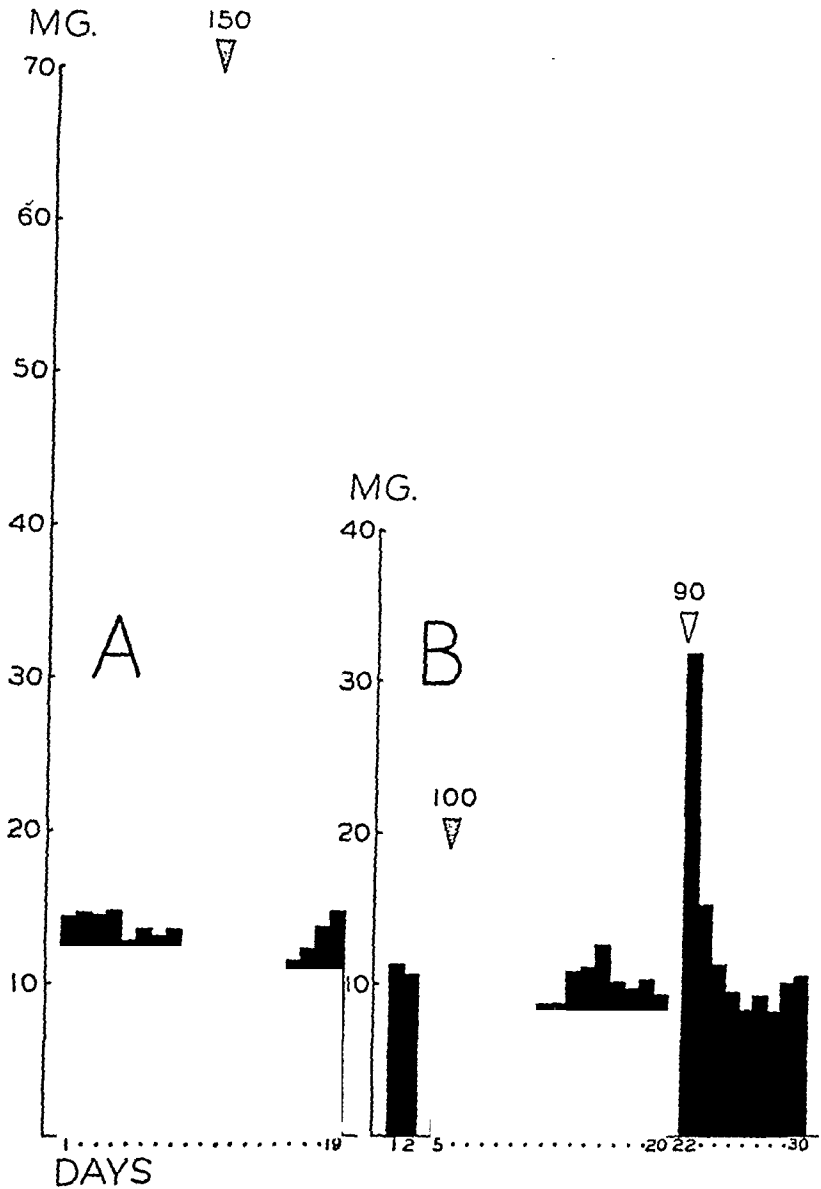


Fig. 1.

*Injections of testosterone propionate and the urinary excretion of 17-ketosteroids in Expt. Nos. 1 and 2. Ordinates: 17-KS in mg. per 24 hours. Abscissae: Days of observation period. The white wedge-shaped triangle indicates the injection of an oily solution, and the dotted triangles indicate the injection of an emulsion. The figures above the triangles show the amount of substance in milligrams.*

breol» emulsion were injected intramuscularly as a single injection. The average daily excretion of 17-KS was 10.8 mg. (calculated from 16 24-hour urines, before the injection and from periods when the effect of this and the following injection had disappeared). The 17-KS content in the first four days after the injection was 18.3, 18.0, 14.0 and 13.7 mg./24 hours, respectively; 37 per cent of the 100 mg. TP injected was excreted as 17-KS, allowing for the hypophyseal inhibition, otherwise the figure was 25 per cent. When the excretion of 17-KS had reached the normal values, 90 mg. of TP in oily solution were given as a single intramuscular injection. An increased 17-KS excretion was found for 3 days, and it was calculated that from 36 to 46 per cent of the TP injected was recovered as 17-KS. In this experimental subject the increased 17-KS excretion lasted for 4 days after administration of the emulsion, and for 3 days after the oily solution, and there was an almost equal excretion on the first and second days after the injection of the emulsion. The utilization of the TP in the form of emulsion seems, however, to have been slightly less than that observed after the injection of TP in oily solution. The local and general reactions observed after the injection of the emulsion were identical with those in the above-mentioned case.

*Expt. No. 3 (Fig. 2, C).* The average excretion was in this case 9.3 mg./24 hours, calculated on the basis of 8 determinations carried out shortly before the first injection and 7 determinations about four weeks after the last injection. At first 150 mg. TP in oily solution were given intramuscularly as a single injection. The 17-KS excretion rose to 46.9 mg. in the first 24-hour urine and remained high for 3 days. The extra excretion was 77.7 mg., corresponding to 62 per cent of the TP injected. On the 5th day he was injected with 150 mg. »Neo-Hombreol« emulsion, and the 17-KS values were 23.5, 19.8, 15.5 and 15.1 mg./24 hours in the following 24-hour urines. The observation was then interrupted, because the experimental subject had to make a trip abroad. The extra excretion in the course of the four days amounted to 36.7 mg.,

that is 29 per cent of the TP injected. This figure is undoubtedly too low, since a considerable hypophyseal inhibition must have occurred as a result of the administration of

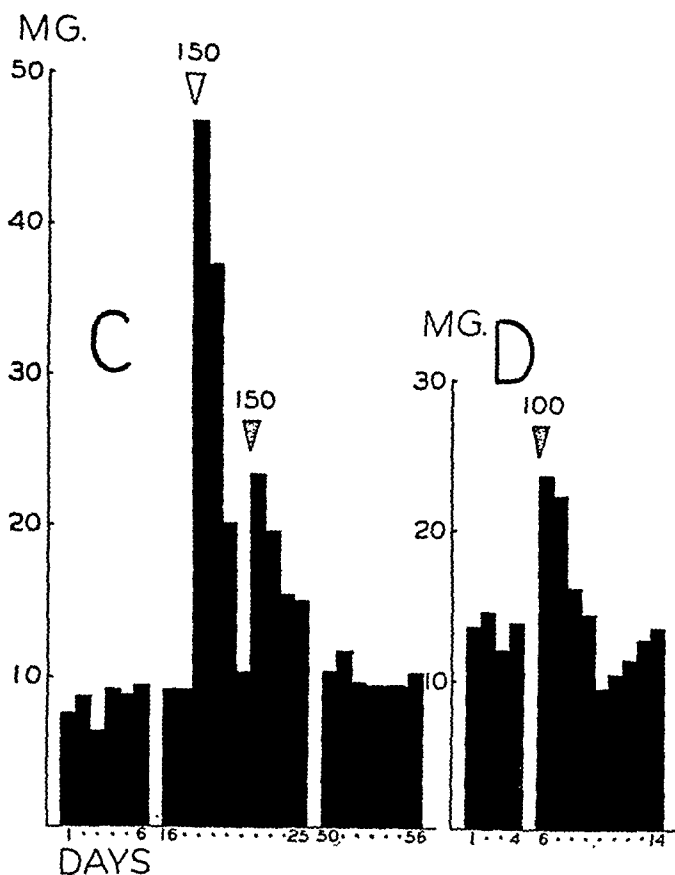


Fig. 2.

*Injectons of testosterone propionate and the urinary excretion of 17-ketosteroids in Expt. Nos. 3 and 4. (For explanation, see legend to Fig. 1).*

300 mg. TP; furthermore it is likely that unabsorbed material was present after the 4th day. In this case the absorption from the deposit of the emulsion was probably slower than from the oil deposit, but final proof is lacking because of the interruption of the experiment. No untoward reactions, either local or systemic, occurred in this case after the injections.

*Expt. No. 4 (Fig. 2, D).* The 17-KS excretion was determined for 4 days prior to the injection of 100 mg. »Neo-Hombreol« emulsion and was on an average 13.5 mg./24 hours. The excretion was increased for 4 days after the injection (23.7, 22.3, 16.1 and 14.4 mg. 17-KS/24 hours, respectively), and a marked hypophyseal inhibition was observed afterwards. The calculations showed that not less than 27 and not more than 46 per cent of the 100 mg. TP injected was excreted as 17-KS. The injections produced no untoward reactions whatever.

### DISCUSSION

The present experiments with testosterone propionate in aqueous emulsion (»Neo-Hombreol« emulsion, Organon) gave quite unexpected results. In all 4 cases the maximal 17-ketosteroid excretion occurred in the specimen of urine collected during the first 24 hours after a single intramuscular injection of 100 or 150 mg. testosterone propionate in emulsion. In this respect the excretion pattern after an injection of an emulsion was similar to that after an injection of an oily solution, and differed markedly from the 17-ketosteroid excretion observed after a single injection of a crystal suspension of testosterone propionate (»Perandren«, Ciba) as previously reported by *Hamburger & Kaae* (1949). In those experiments the maximal excretion occurred on the 3rd to the 5th day after a crystal injection.

In three of the present cases the difference between the 17-ketosteroid excretion on the first day and on the second day was, however, smaller than is usually observed after oily injections, only in the first experiment was it as high as after an oily injection.

As to the duration of the period of increased 17-ketosteroid excretion, no great differences were observed between the emulsions and oily solutions. In three of the cases the increased excretion lasted for only 3 or 4 days, whereas an increased 17-ketosteroid excretion lasted for an average of 12 days after a single injection of a crystal suspension of



»Perandren«. In one of the experiments the observation was interrupted before the excretion had fallen to the normal level.

Although limited in number, these experiments show that as far as the 17-ketosteroid excretion and hence also the absorption rate, after a single intramuscular injection are concerned, the »Neo-Hombreol« emulsions must be placed between

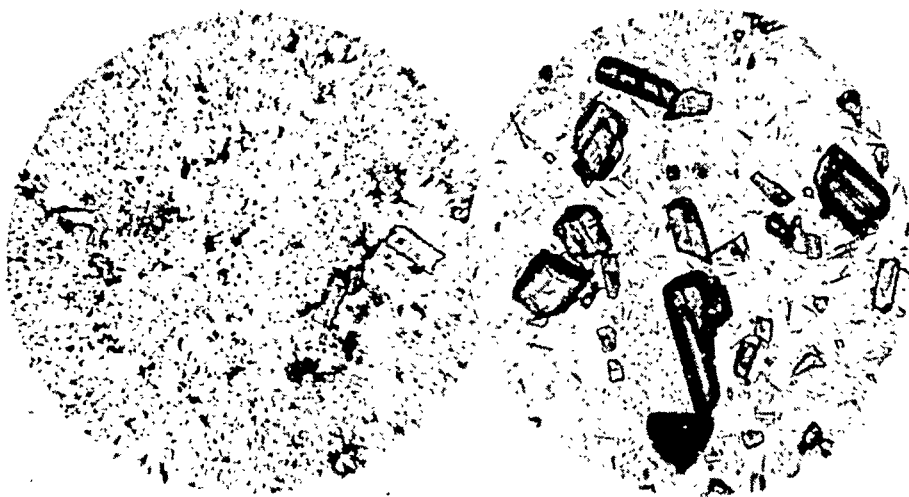


Fig. 3.

*Left: Crystals of testosterone propionate two hours after dilution of the emulsion with water. Right: Crystals from a »Perandren« crystal-suspension ampoule. Same magnification ( $\times 50$ ).*

»Perandren« crystal suspensions and the oily solutions, and certainly closer to the latter.

How can these disappointing results be explained? Let us consider first the absorption of testosterone propionate from an intramuscular oily deposit. The oil presumably spreads out into thin films between the muscle fibres. From these oily films the hormone is absorbed, leaving the oil itself unabsorbed. This absorption is a very rapid process, since an appreciable amount of testosterone propionate is excreted as 17-ketosteroid in the urine within a few hours of the injection.

In the aqueous emulsion the hormone is dissolved in the extremely small globules of benzyl alcohol, and it is reasonable to assume that an appreciable absorption of hormone takes place from these globules (with their relatively large surface) before any precipitation of crystals sets in. This might explain the high 17-ketosteroid excretion in the first 24-hour urine, as observed particularly in expt. No. 1.

The absorption from the crystals, escaping this initial absorption, depends on their size. When the formation of crystals in the emulsion of »Neo-Hombreol« after dilution with water is observed under the microscope, it is seen that the precipitation is no immediate process. In the course of about a quarter of an hour distinct small crystals have formed. A comparison of the crystals, as observed 2 hours after the dilution of the emulsion, with the crystals in a commercial »Perandren« crystal-suspension ampoule, is shown in Fig. 3. Most of the crystals from the emulsion are minute granules and only few larger rectangular crystals are found, whereas the »Perandren« preparation contains several large crystals and some fine needles.

Information concerning the process of crystallization in human muscular tissues is obviously desirable, but our experiments seem to indicate that the crystals formed in man might, on an average, be smaller than those present in the commercial »Perandren« preparations. It is unlikely that the actual dose used in the present experiments (100 or 150 mg., instead of the proposed clinical dose of 50 mg. (*Lens et al.*, 1949)) has had any significant effect on the results obtained.

### SUMMARY

Single intramuscular injections of testosterone propionate in aqueous emulsion were given to four healthy young and middle-aged men.

Determinations of the urinary excretion of 17-ketosteroids revealed that the hormone was absorbed from the emulsion injected at a rate somewhat slower than from oily solutions,

but much faster than from a commercial crystal-suspension preparation.

It is assumed that some absorption occurs from the emulsion before the crystallization has taken place, and that the crystals formed in the human muscular tissues are, on the average, smaller than those present in the commercial crystal-suspension preparation.

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**COPULIN AND OVIPOSITOR GROWTH  
IN THE FEMALE BITTERLING (RHODEUS  
AMARUS BL.)\***

BY

B. DE GROOT and J. J. DUYVENÉ DE WIT

**INTRODUCTION**

In 1939 one of us (D. d. W.) recorded the results of experiments on the induction of ovipositor growth in the bitterling. A number of male bitterlings in display — without either females or mussels — were kept in an aquarium for a few days. The males were then removed, and the water transferred to a number of small tanks after which three female bitterlings were placed in each tank. These females had previously been sensitized, i. e. they had been treated with the urine of pregnant women, after which their ovipositors had again become shortened to a few A. U. (A. U. is an abbreviation of »anal fin unit«, and corresponds to one-eighth of the foremost radius of the extended anal fin). Within one hour there was marked growth of the ovipositors (see Fig. 1), and it was evident that the males had secreted into the water a substance which — as was subsequently proved by

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\*) 28th communication of the »Werkgemeenschap voor Endocrinologie«, part of the »National Council for Agricultural Research T. N. O.«.

histological examination of the ovaries — exerted a direct action on the ovipositor tissue. Following *Jaski's* investigations (1939) this substance was called *copulin*, and regarded as a »male-fish-hormone« which differs from the mammalian steroids in that it acts directly on the ovipositor and not via the hypophysis.

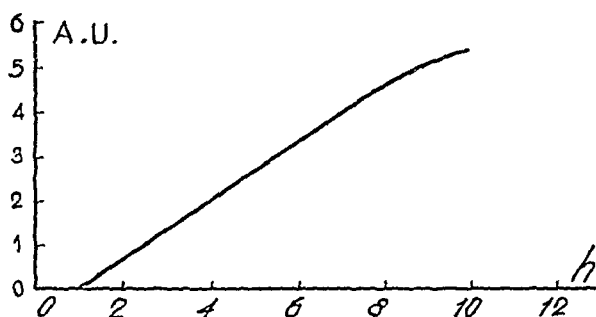


Fig. 1.

Ovipositor growth curve obtained in water, activated by bitterling males (from *Duyvené de Wit*, 1939).

Recently *Van Koersveld* (1949) has failed to confirm these results and has stated that under experimental conditions he was unable to demonstrate the production of copulin by male bitterlings. Further he has expressed the strongest doubts as to the existence of this substance. According to him, the effects obtained by *Duyvené de Wit* were probably due to a pollution of the water, and he further suggests that ovipositor growth in the bitterling is usually due to toxic substances formed during organic decay. This contention decided us to repeat once again our experiments of ten years ago, under the most carefully controlled conditions.

## EXPERIMENTS

In the spring of 1948 we had at our disposal a collection of bitterlings, of which most of the males, as shown by their nuptial colours, were in display. Eighteen male fishes were distributed in 6 small aquaria, each containing 750 ml. water at 23° C, so that there were 3 in each tank. The water was

well aerated, and the fishes were fed on set quantities of mosquito larvae. The water was sieved daily to remove excreta. In spite of this the water became slightly turbid in the course of the week.

After 7 days the males were replaced by unsensitized females with short ovipositors. Within a few hours a significant ovipositor growth was observed (see Fig. 2, Curve A). This experiment was later repeated twice, each time with a positive result.

In order to disprove still further *Van Koersveld's* contention that ovipositor growth is related to the accumulation of (bacterial) waste products in the water, the experiment was repeated with female instead of male bitterlings. The 18 females were kept for a week in precisely the same conditions as the males in the previous experiments. They received the same nourishment, and their water was also sieved to remove the excreta. When after 7 days these females were replaced by other, unsensitized females with short ovipositors, no ovipositor growth occurred.

It is thus evident that only males secrete a substance causing ovipositor growth, and that this effect cannot be due, as *Van Koersveld* claims, to unspecific, toxic (bacterial) metabolic products, accumulating in the water during the experiments.

That the secretion of copulin is, in effect, a vital process receives additional support from the following considerations.

According to *Selye* (1948) an adaptation to a non-specific, noxious stimulus takes place at the expense of other specialized physiological functions. It appeared possible that the production of copulin is one of these specialized functions, which — under the influence of a harmful stimulus — would be reduced or completely interrupted.

To test this, a number of displaying male bitterlings were taken, and kept for a week in the same conditions as those described above except that they were exposed to strong light. The harmfulness of this stimulus was manifested when the fishes assumed a darker colour — a phenomenon which in-

variably occurs when bitterlings are exposed to a noxious agent, and which must accordingly be regarded as one of the signs of the adaptation syndrome. After 7 days unsensitized females were introduced into the tanks. The reactions of their ovipositors are recorded in Fig. 2, Curve B. This shows that

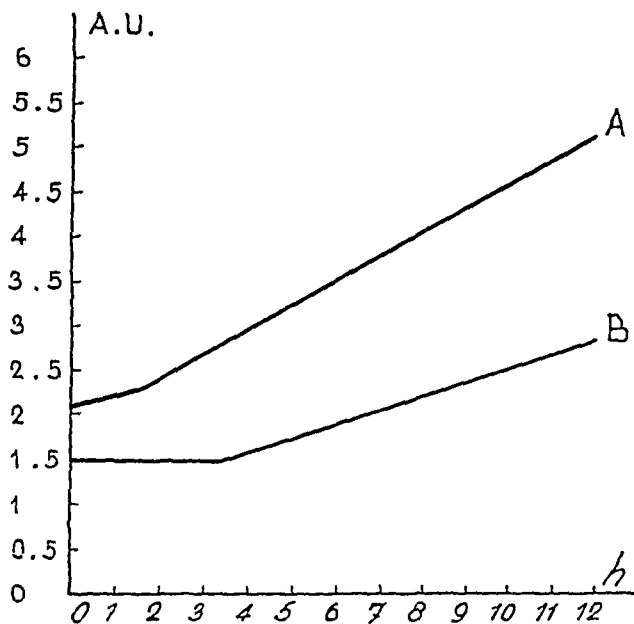


Fig. 2.

Ovipositor growth curves obtained in water activated by bitterling males.

A: under normal light conditions. B: under the influence of continuous strong lighting (»damaging agent«).

the water contained less copulin than it would have done if the light-stimulus had been omitted. In agreement with *Selye's* theory, this would be explained by assuming that the adaptation to the damaging agent acts as a drain on the energy normally expended on such processes as the production and secretion of copulin.

In all the above mentioned experiments, the sensitivity level of the females was regularly controlled by causing corresponding fishes to react to  $8 \gamma$  progesterone, while in addi-

tion a »blank« parallel test was made in each case as a general control.

### DISCUSSION

Recent investigations have suggested that the social relations between fishes of the same species, as well as those between the sexes, are more complex than was formerly believed. We need only think of the social significance of smell and taste, including the perception of substances causing fright reactions, and that of the production and appreciation of species- and sex-specific sounds. On the basis of the above experiments the question immediately arises as to whether we are also justified in attributing a biological significance to the excretion of copulin.

With regard to this question it must first be mentioned that the rhythmic and cyclic ovipositor growth of the bitterling, which was carefully investigated by *Meltzer* (1947) (see Fig. 3), occurs only when both a male bitterling and a mussel are present. If one or both of these are absent then the ovipositor stays at a length of about 5 A. U., and there is no oviposition. Evidently stimuli emanating both from the male fish and from the mussel dominate the growth cycle of the ovipositor, and cause oviposition every 6th—8th day. On this day the ovipositor suddenly grows to a length of 2—3 cm., and eggs are repeatedly laid. It is not yet known whether these stimuli are of an optical or chemical nature.

When the male bitterling is ready to display at spawning time, it seeks a mussel, which then forms the centre of its territory. From this territory fishes of the same and other species are continually chased away. Not only are males driven off, but even females with long ovipositors. Those females, however, which, apart from possessing a long ovipositor, also adopt a certain posture, namely, with the head inclining slightly downwards (inclination position) appear to attract the special attention of the male. These females do not take fright as the male rushes towards them impetuously, but rather con-



vey the impression of being apathetic. When a male encounters such a female his behaviour suddenly changes. Displaying himself, and trembling all over, he slowly swims before her in the direction of the mussel. The female seems fascinated by these tremblings, and follows slowly until she comes to the mussel. There she inspects the filaments of the exhalent siphon, and if the conditions are satisfactory places herself

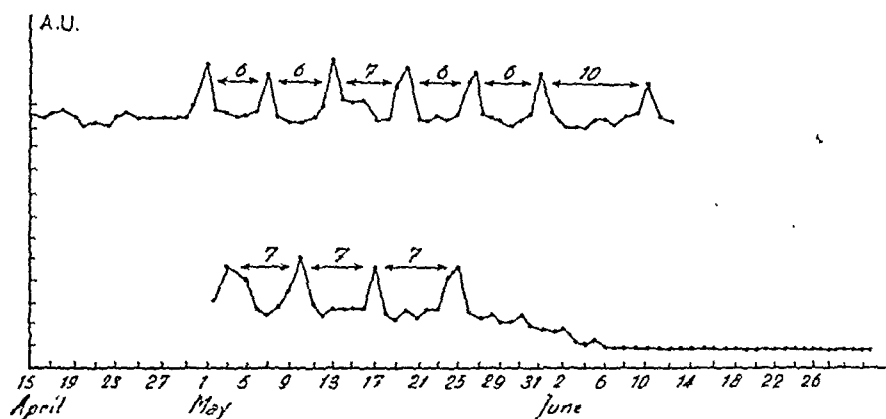


Fig. 3.

Periodical fluctuations in ovipositor growth in the female bitterling during spawning time. Repeated oviposition occurs at each of the peaks (from Meltzer, 1947).

with the tail above the siphon, her head slightly downwards. Then with a sudden forward movement she shoots her ovipositor into the opening. During this manoeuvre, which lasts only a fraction of a second, the displaying male performs extremely rapid trembling movements, which probably constitute the actual oviposition stimulus to the female. As soon as the latter has deposited her eggs and has swum away, the male, shining in brilliant colouring, discharges his sperm from above into the same opening.

Since bitterlings prefer to spawn in stagnant or very gently flowing water, it is quite possible for the territory of the displaying male to be impregnated with copulin, even if this is

present only in very weak concentrations. Females which, in different phases of their sexual cycle, are continually cruising through such territories, apart from receiving an optical impression of the male and of the mussel, will also be able to absorb small quantities of copulin. That even a very short period of hormonal action may influence the bitterling is evident from the fact that females, swimming for only 5 minutes in a very weak progesterone solution (say 20  $\gamma$  per litre) may show ovipositor growth within a few hours. Probably the epithelium of the gills is readily permeable to substances of this nature. Thus it seems that in natural conditions the cyclic ovipositor growth of the female bitterling may be the result of the *combined* actions of optic stimuli and of stimuli arising from the repeated absorption of small quantities of copulin, entering indirectly through the gills and perhaps also directly through the ovipositor. This view led *Bretschneider et al.* (1941 a, 1941 b, 1947) to speak of a »doppelte Sicherung« (a double assurance) by which optimum conditions could be maintained for cyclic ovipositor growth, and so, in the last analysis, for the maintenance of the species. Further investigation of this point is, however, needed.

### SUMMARY

In contradiction to the findings of *Van Koersveld* (1949) further carefully conducted investigations have confirmed the previous observation that, during the spawning season, the male bitterling secretes a substance into the water, which promotes ovipositor growth in the female. This substance has been named copulin.

Under the influence of a damaging agent (in this case constant strong light) the production of copulin was found to decrease.

The possible significance of this hormonal substance during the spawning season of the bitterling is discussed.

### Acknowledgement.

We are greatly indebted to Dr. W. S. Bullough for his assistance and valuable advice during the preparation of the manuscript.

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## HYALURONIDASE CONTENT OF TESTES IN RATS OF DIFFERENT AGES

BY

OVE RIISFELDT

During recent years investigations on the enzyme hyaluronidase have been concerned with its chemical properties and its importance in physiology and pathology. However, in the growing literature on the subject none of the more comprehensive studies deal with the origin of the enzyme, i. e. to which cells in spermatogenesis the formation of hyaluronidase is related. This question will be discussed in the following.

Based on initial investigations by *Duran-Reynals* (1928, 1929), *Hoffman & Duran-Reynals* (1930, 1931) and at the same time *McClean* (1930) demonstrated that mammalian testes contained a factor, the spreading factor, which was capable of increasing the permeability of connective tissue. This was shown by giving a rabbit an intracutaneous injection of testicular extract; the bleb area thereby produced became larger than when the same amount of extraction fluid alone was used for the injection. The diffusion was indicated by the addition of Indian ink. Since the spreading in the tissue was thus increased by addition of the testicular extract, the factor causing the diffusion was termed the spreading factor, or later *Duran-Reynals* factor.

This spreading factor has not only been observed in testicular tissue, but also in snake venom (*Duran-Reynals*, 1939); in bacteria (*Duran-Reynals*, 1933; *McClellan*, 1936); in leeches (*Claude*, 1937); and in placental tissue and malignant tumours (*Duran-Reynals & Steward*, 1931; *Boyland & McClellan*, 1935).

Independent of these investigations, *Meyer & Palmer* (1934) succeeded in isolating hyaluronic acid from vitreous humour, synovial fluid, and human umbilical cords. It appeared to be a high-molecular polysaccharide, a compound consisting of glucuronic acid and N-acetyl-glucosamine. Hyaluronic acid can be split by enzymes in the autolysate of pneumococci, iris, etc. (*Meyer et al.*, 1940), and *Chain & Duthie* (1939, 1940) showed that the spreading factor was also capable of decomposing hyaluronic acid, and that the spreading factor was identical with a hyaluronidase.

### IMPORTANCE OF HYALURONIDASE

Here it will be sufficient briefly to mention that according to previous investigations hyaluronidase is of definite importance at least in fertilization, the hyaluronidase content of human semen being roughly proportional to the number of spermatozoa (*Joël & Eichenberger*, 1945; *Werthessen et al.*, 1945; *Kurzrok et al.*, 1946; *Riisfeldt*, 1949 a). *McClellan & Rowlands* (1942) showed that hyaluronidase was capable of dissolving the substance which keeps the cumulus and corona radiata cells around the ovum together; thus these cells are detached, so that the spermatozoa can penetrate the egg.

The production of hyaluronidase by bacteria is supposed to account for their invasion of the organism in infectious disease (*McClellan & Rogers*, 1943). Incidentally, a comprehensive survey of these conditions has been published by *Duran-Reynals* (1942). In cancer research the importance of hyaluronidase in the invasion of cancer and the occurrence of metastases has been considered (*Riisfeldt*, 1949 b), but the question, which is as yet rather obscure, will not be discussed here.

## DETERMINATION OF HYALURONIDASE

Several methods, both biological and physicochemical, have been employed in the determination of hyaluronidase.

*A. Biological Methods.*

1. The biological method was the one by which *Duran-Reynals* originally demonstrated the existence of hyaluronidase. Since then several modifications have been elaborated (*Bacharach et al.*, 1940; *Humphrey*, 1943), but they generally follow the original principle of measuring the size of the bleb area produced after the lapse of a certain time after the intracutaneous injection of the enzyme-containing material and comparing it with the size of the bleb produced by the injection of the extraction fluid alone.

2. *Leonard & Kurzrok* (1946) have used the rat-ova test based on the observation of *McClean & Rowlands* (1942) that hyaluronidase is capable of dispersing the cumulus cells and the corona radiata; they measured the reaction time, i. e. until complete denudation of the egg had taken place.

*B. Physicochemical Methods.*

1. Viscosimetry. When *Chain & Duthie* (1939, 1940) had shown that the spreading factor was identical with a hyaluronidase, *Madinaveitia & Quibell* (1940) devised a method for determining hyaluronidase by means of viscosimetry. The method is based on the fact that hyaluronic acid in solution is very viscous, which is not the case with its breakdown products. Subsequently, other viscosimeters capable of dealing with the small amounts of material available have been described, e. g. by *Werthessen et al.* (1945) and *Dalgaard-Mikkelsen & Kvorning* (1948).

2. »Mucin clot prevention test«. *Seastone* (1939) found that hyaluronic acid produced a typical mucin clot when it was mixed with serum protein and acetic acid was added. *Robertson, Ropes & Bauer* (1940) utilized this reaction in the determination of the mucin-splitting enzyme in clostridium *Welchii*, and *McClean* (1943) further elaborated the method.

3. Turbidimetry. This method of measurement, which has been worked out in detail by *Kass & Seastone* (1944) and *Leonard et al.* (1946) is based on the same principle as the »mucin clot prevention test«, but the determination is made by means of a colorimeter.

4. Determination of the breakdown products of the hyaluronic acid has been carried out by *Meyer et al.* (1940, 1941) and *Rogers* (1946). These determinations are, however, too complicated to be used as a routine.

### HYALURONIDASE CONTENT OF TESTES

Few investigations on the hyaluronidase content of testes at different stages of the development are available in the literature. *Sprunt et al.* (1939) appear to have been the first to have concerned themselves with this problem, and they found smaller amounts of the spreading factor in the testes of cryptorchid and young rats than in those of adult animals. *Leonard et al.* (1948) showed that testes from 21-day-old rats contained only very little hyaluronidase, while testes from 50-day-old rats contained almost the same amounts as those from 90-day-old rats. In a preliminary report *Riisfeldt* (1949 c) described similar results, of which a detailed account is presented here.

### OWN INVESTIGATIONS

*Viscosimetry.* The amount of hyaluronidase has in all cases been determined by means of the viscosimetric technique. The viscosimeter used (Fig. 1) is a modified Ostwald viscosimeter which has been devised and made by Dr. *Holm-Jensen* for the present purpose. It differs from the usual Ostwald viscosimeter in that the mean value of the hydrostatic pressure of the fluid in the viscosimeter during the outflow is zero.

*Substrate.* As substrate a hyaluronic-acid preparation is used which is made from umbilical cords according to the method of *Meyer & Palmer* (1934). The substrate obtained is readily water-soluble and has a considerable viscosity. It has been used in a solution which is approximately three times as viscous as water.

*Hydrogen-Ion Concentration and Buffer.* Like all other enzymatic reactions this is markedly affected by the hydrogen-ion concentration. *Hale* (1944) found the optimum reaction at pH 7.0 when a 0.1 M citric acid phosphate buffer was used.



Fig. 1.

The viscosimeter. (The volume between the upper and lower marks is 2.5 ml.).

*Temperature.* Both the viscosity and the velocity of the enzymatic process are dependent on the temperature, which must therefore be kept constant. Previous investigators have used various temperatures in their measurements; thus *Eichenberger* (1946) 20° C. and *Hale* (1944) 34° C. In the study presented here a temperature of 37° C. was chosen since at this temperature the reaction occurs at a suitable velocity and is in conformity with physiologic conditions. The whole viscosimeter was therefore placed in a water-bath at this temperature.



*Determination of the Flow Time.* The viscosimeter is connected with a water manometer, so that the flow through the viscosimeter takes place at a constant pressure determined at will. By means of rubber tubing with an inserted stopcock, the viscosimeter is connected with a ten-litre flask containing approximately one litre of water. The manometer tube passes through the stopper of the flask and opens under the surface of the water. Through the stopper a pressure pump is connected with the flask, so that the pressure can be regulated. This pressure is read as centimetres of water on the graduated manometer tube. When the stopcock to the viscosimeter is opened, the fluid is forced through the viscosimeter, and the time needed for the fluid surface to pass the two marks is read. When the lower mark on the viscosimeter has been passed, the rubber tubing is moved to the reservoir of the viscosimeter (the wide tube), so that the fluid is forced back through the viscosimeter and is allowed to rise above the upper mark, and the determination is repeated. The entire arrangement is shown in Fig. 2.

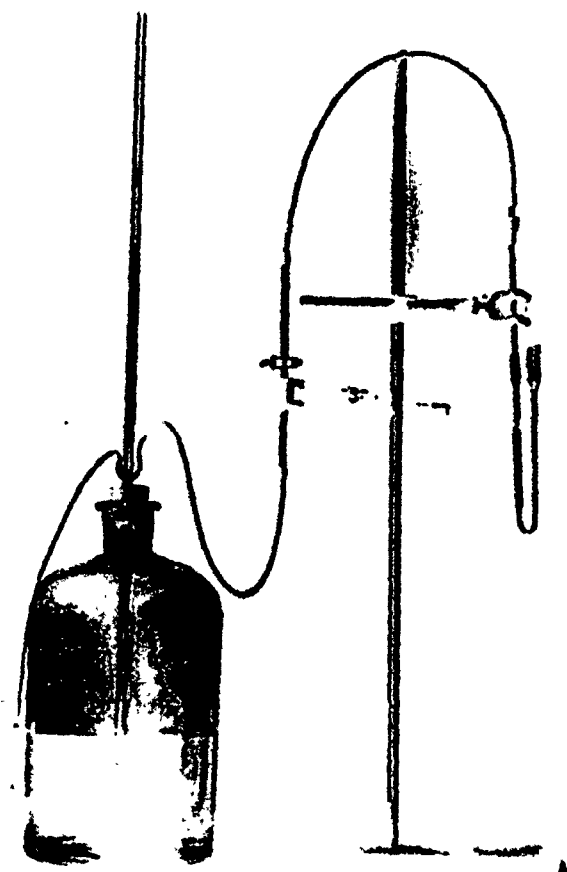
A pressure of 20 cm. water was found to be suitable for the determinations, and hence this pressure was used in all cases. The flow time thus became 29 seconds.

*Hyaluronidase Determination.* To 4.0 ml. substrate solution 0.5 ml. citric acid phosphate buffer is added and the preparation is heated in a water-bath to 37° C.

One half millilitre of the enzyme solution to be examined is pipetted off, and as soon as it is added to the substrate-buffer solution, a stop watch is started. After having quickly shaken the test tube with the solution, 4 ml. of it are pipetted off into the wide tube of the viscosimeter, which is immediately put under pressure from the manometer flask, and the fluid is forced through the viscosimeter till the surface is a little above the upper mark, and the measurements are carried out as described above. In this way a consecutive series of determinations of the flow time is made. The times at which the flow time is determined are read, and the time of the mixture of

the enzyme solution and the substrate-buffer solution is taken as the starting point.

Owing to the change of viscosity during the flow time a correction for this must be made. This is done by adding one half of the flow time to the time elapsed when the determination begins.



*Fig. 2.*

The entire arrangement for the viscosimetry.

The relative viscosity (as compared with water) is computed, and it is then plotted against the time from the commencement of the experiment until each individual determination has been made with the correction mentioned above, the relative viscosity and time being used as ordinate and abscissa respectively. In most cases the initial value can be determined

by boiling the enzyme solution, so that the enzyme is destroyed, and the viscosity is then measured in the usual way. This method can be used in the great majority of cases since after having been centrifuged the solutions will be practically protein-free, and there is no precipitate on boiling. In the cases where the fluid cannot be completely freed from protein, the initial value must be computed by extrapolation. Fig. 3 shows an example of a curve of the hyaluronidase activity of a protein-free solution. The half-viscosity level is computed, i. e., the time needed for the enzyme to reduce the viscosity to one half of the original value.

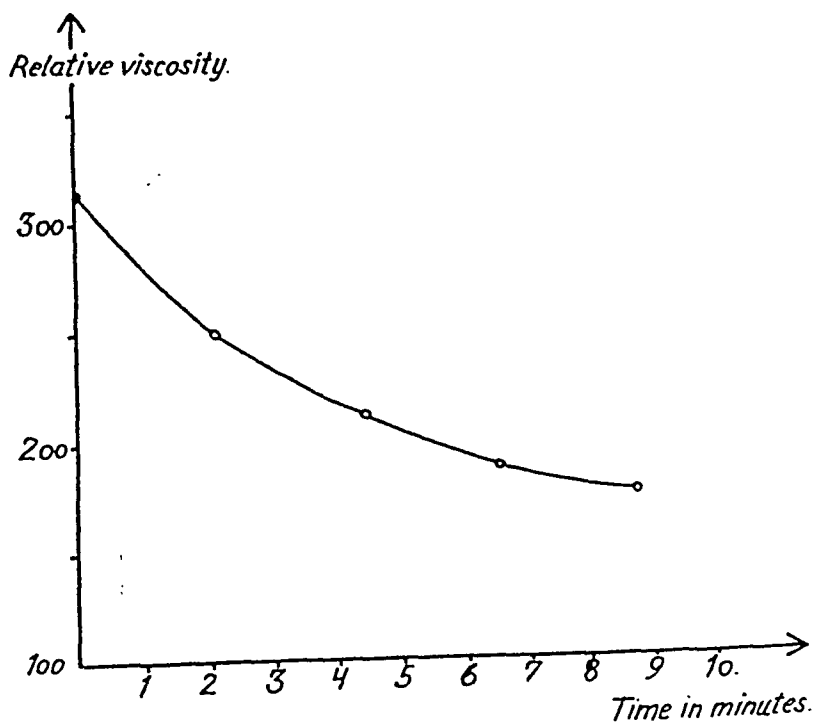


Fig. 3.  
Hyaluronidase activity of a diluted and centrifuged testicular extract.

The half-viscosity level is obtained by the intersection point of the curve and the line indicating one half of the original value of the relative viscosity.

So far various units have been used to indicate the hya-

luronidase activity, and an international standard is still not available. *McClellan & Hale* (1941) used a viscosity reducing unit (V. R. U.) which they defined as the amount of enzyme capable of reducing the viscosity to one half of the original value in the course of 20 minutes. In measurements of the hyaluronidase activity by means of turbidimetric technique, turbidity reducing units have been used, but other units have also been employed, e. g. by *Dalgaard-Mikkelsen & Kvorning* (1948).

In the present study the hyaluronidase activity is measured in V. R. U., since here it is not so much a question of giving the absolute as the relative amounts found in the various testicles, and for this purpose one unit may be just as good as another, especially as the initial value can be determined by the viscosimetric assay, which is not always the case in the determination of the hyaluronidase activity in other organic materials.

### MATERIAL

In the investigations reported here 69 Wistar rats of an age varying from 15 days to about six months have been used. They were all normal animals which had not been used in other experiments, and they had been given the usual food for laboratory animals.

*The procedure* was as follows: The animal was weighed, killed, and both testicles were immediately removed and weighed separately. The tunica vaginalis propria was then removed, so that only the pulpal tissue remained. A piece of the pulpal tissue was removed for microscopic examination, and the rest was weighed. Admixture of blood was carefully avoided in order to prevent the determination from being affected by the hyaluronidase-inhibiting property of the blood.

The weighed pulpal tissue is placed in a mortar and ground with a little sand in order, as far as possible, to destroy the cell membranes and release the tissue fluid. During the grinding a quantity of water equal to ten times the weight of the pulpal tissue is added. After thorough stirring the prepara-

tion is allowed to stand undisturbed in the ice-box for 24 hours at 4° C., so that the extraction can be as complete as possible without damaging the hyaluronidase.

When the extraction is completed, a further dilution up to 50—100 times the original quantity of pulpal tissue is made in those cases in which there is a considerable hyaluronidase content in the extract. This dilution is necessary; otherwise the entire content of hyaluronic acid of the substrate will be split at once, so that the characteristic curve shown in Fig. 3 is not produced.

The diluted extract is then centrifuged till the supernatant fluid is completely clear, and this fluid is used for the viscosimetric assay. A few millilitres of the fluid is heated to boiling point and used as the initial value in the computation of the hyaluronidase activity.

## RESULTS

The results of the experiments appear in Table 1. Instead of stating the exact age of the rats the histologic picture of the testicles in the individual cases has been stated, groups of rats of the same age having been selected so that the histologic picture is as uniform as possible.

In Group I where there were only spermatogonia in the testicles it proved impossible to demonstrate the presence of hyaluronidase in measurable amounts. As the individual testicles were very small in this group, all the testicles from five rats were used in each experiment in order to obtain a sufficient amount of extract for the determination, and the preparation was diluted with water only five times the weight of the pulpal tissue. In the viscosimetric assay the initial value was obtained by extrapolation as the extract contained some protein, so that it could not be boiled without giving disturbing viscosimetric changes.

By the microscopic examination in Group I only spermatogonia were found, which shows that functionally at least it is a resting tissue.

In Group II spermatogonia and a few spermatocytes were

found. It was just possible to demonstrate the presence of hyaluronidase, but not in such quantities that it could be measured by the method used.

*Table 1.*

Relation between histologic examination of the rat testes and the content of hyaluronidase.

Group	Number of rats	Histologic examination	Hyaluronidase V.R.U. pr gm. wet tissue	
			Range	Mean value
I	20	Only spermatogonia	0	0
II	8	Spermatogonia and a few spermatocytes	just demonstrable	
III	8	Increasing number of spermatocytes, incipient formation of tubules, no lumina	6—18	13
IV	7	Large number of spermatocytes, plus tubules, no lumina	65—96	89
V	6	Many spermatocytes, small lumina, no spermatozoa	115—141	134
VI	6	Almost complete spermatogenesis in the whole testis and a few spermatozoa	158—186	174
VII	14	Adult rats with normal spermatogenesis	164—210	184

In Group III a larger number of spermatocytes was present, and formation of tubules with stratified epithelium, but no distinct lumina were seen. The hyaluronidase content was increasing, although it was still present only in very small amounts.

In Group IV the number of spermatocytes had again in-

creased, but no spermatozoa were seen. Distinct tubules were present, the epithelium had become high, and lumina were also visible. The hyaluronidase content was now considerable.

In Group V the tubules and lumina were distinct, the epithelium of the tubules was high, but no spermatozoa were seen in the lumina. The hyaluronidase content was very considerable, approaching the values found in the fully developed testicle.

In Group VI spermatogenesis was complete; a few spermatozoa in the lumina showed that it had just commenced. The cell proliferation was intense, but the formation of spermatozoa was still rather sparse. The hyaluronidase content in this group came very near to that of Group VII, which represents the fully developed testicle, and it is seen that the values of the hyaluronidase activity in the two groups overlap.

Group VII showed the picture of the fully developed rat testicle with many spermatozoa in the lumina, and here the hyaluronidase content showed the maximum values.

## DISCUSSION

From Table 1 it is seen that the maximum content of hyaluronidase is not present until mature spermatozoa have developed. However, in Group V where there was already a large number of spermatocytes, a considerable hyaluronidase content was found. Compared with Group I, where spermatogonia but no demonstrable amounts of hyaluronidase were present, the experiments show that the presence of hyaluronidase in demonstrable amounts coincides with the occurrence of spermatocytes, and that it increases sharply with the number of spermatocytes.

The formation of hyaluronidase increases rapidly during development. The explanation may be either (1) that the content of the individual cell is constant and the number of cells rapidly increases, or (2) that the content of the individual cell increases with the number of cells during development. Judging from the experiments presented here it is impossible to say which of these explanation is correct, but they show

that an active formation of spermatocytes causes an increase of the hyaluronidase content which is not definitely dependent on the number of mature spermatozoa. This appears from Groups VI and VII; Group VII shows a very marked proliferation with many spermatozoa, while in Group VI there are only a few spermatozoa in spite of a marked proliferation.

The considerable range seen in the fully developed testicles in Group VII is presumably due to the same conditions, since the intensity of the proliferation varies to some extent in the different testicles.

### SUMMARY

A viscosimetric method for the assay of the hyaluronidase content is described. The hyaluronidase content of rat testicles is determined. It appears that the hyaluronidase content increases with the number of spermatocytes. The increase can be demonstrated even before further division of the spermatocytes occurs, and is very considerable before mature spermatozoa are found.

This investigation has been carried out with the support of Kong Christian den Tiendes Fond, to which I beg to offer my sincere thanks.

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## THE DISTRIBUTION OF ORALLY ADMINISTERED RADIO-ACTIVE METHYL- THIOURACIL IN COCKERELS\*

BY

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and W. TH. NAUTA

Since the derivatives of thiourea were discovered to possess antithyroid activity, questions of absorption, retention and elimination of these substances have been repeatedly investigated. For some reason, however, the compound 4-methyl-2-thiouracil, though known to be a powerful anti-thyroid drug, has so far escaped attention in this respect.

Furthermore the investigators who worked in this field (Danowski, 1944; Williams, 1944; Christensen, 1946) invariably based their analyses on a colorimetric determination by means of Grote's reagent. In a single case (Pipes & Turner, 1947) a biological test, based on the increase in weight of the thyroid gland of rats, was used. We have tried to study the distribution of the above-mentioned compound after oral ad-

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ministration by making use of a preparation containing radioactive sulphur, obtained in the following way.

The active sulphur, which was received in the form of a solution of sodiumsulphate, was precipitated, after appropriate dilution with inactive sulphate, as bariumsulphate. The precipitate was centrifuged off, washed and dried, and reduced to bariumsulphide by heating with carbon at a temperature of  $1000^{\circ}$  C., according to a method described by *Peters et al.* (1947). The sulphide was dissolved in a solution of bariumhydroxyde, saturated previously with hydrogensulphide. To this solution cyanamide was added, and the mixture was kept in a waterbath at  $50^{\circ}$  C. for two hours. After the reaction was complete, the barium was precipitated by means of carbondioxyde, and the bariumcarbonate was removed by filtration. The filtrate was dried, and the thiourea thus obtained was converted into 4-methyl-2-thiouracil in alcoholic solution according to *Wheeler & McFarland* (1909). The alcohol was distilled off, the residue dissolved in water and the methylthiouracil precipitated by means of acetic acid. After being washed and dried the preparation was analyzed according to »Analysmetoder Nordiska Specialitets Kommissionen (Häfte 16, 1946)« and turned out to have a purity of more than 98 per cent. Finally the activity was brought to the desired value by dissolving the substance together with the appropriate amount of inactive methylthiouracil in ammonia and reprecipitating with acetic acid.

We have tried to get information on the following points:

1. The distribution of a single dose in the different organs.
2. The time needed for complete excretion of a single dose.
3. The occurrence of a cumulative effect after repeated dosage.

As experimental animals cockerels were used, about 15 weeks old on an average. The animals were kept in cages with a wire netting bottom over a glass plate, in order to be able to collect the excreta quantitatively. The drug was administered by means of tragacanth pills, each containing 50 mg. of the radio-active preparation. In all cases weighed samples of the organs (about 250 mg.) were collected immediately after the animals had been killed.

The samples were heated with 2 ml. of nitric acid in Carius-tubes in an electric oven at 275° C. for 3 hours. The resulting liquid, in which the sulphur was converted completely into sulphuric acid, was removed quantitatively to a glass-beaker. The excess of nitric acid was removed by evaporation and after the liquid had been diluted, the sulphuric acid was precipitated as bariumsulphate. The precipitate was collected quantitatively and dried, and its activity was measured under a mica window counter.

To be able to convert these measurements into mg. of methylthiouracil, we determined the amount of radio-active sulphur in the preparation used in exactly the same way. It is clear that by this method we not only determine the amount of methylthiouracil in the organs, but also all its decomposition products. We have tried to effect a separation by using a method for the extraction of methylthiouracil, but this did not lead to satisfactory results. Therefore the values given below as amounts of methylthiouracil possibly include small amounts of the decomposition products.

Table 1 contains the results in mg. of methylthiouracil per gm. of tissue. The first column shows the figures obtained after giving a single dose of 50 mg., the animals having been killed 24 hours later. In the next two columns the dose is the same, but the animals have been killed after a longer period, 48 and 96 hours respectively, in order to establish the time needed for complete disappearance of the substance from the body. The rest of the table relates to the cumulative experiments, in which 3, 6 and 9 daily doses of 50 mg. were given, the animals being killed in all cases 24 hours after having received the last dose.

The figures clearly show, that after 24 hours the fraction of the active substance not yet excreted is more or less uniformly distributed in the different organs. Only the thyroid gland, the pituitary gland and the bases of the feathershafts show concentrations which are distinctly above the average. The high content of the thyroid gland was to be expected to some extent, considering the specific action of the compound

Table 4.

Amounts of methylthiouracil in per mille after different treatments.

Number of doses	1 active			3 act.	6 act.	9 act.	8 inact. 1 act.
Interval (hours)	24	48	96	24	24	24	24
Thyroid gland	0.184	0.089	0.012	0.211		0.412	0.431
Pituitary gland	0.020			0.034	0	0.059	0.031
Feathers	0.006	0.006	0	0		0.008	0.003
Shaftbases	0.012	0.009	0	0.023		0.036	0.032
Muscles	0.003	0.008	0	0	0.003	0.007	0.003
Blood	0.011			0.003	0.003	0.012	0.003
Spleen	0.003			0.003	0.009	0.011	0.009
Liver	0.004			0.004	0.005	0.010	0.016
Kidney	0.012			0.004	0	0.019	0.009
Adrenals	0			0	0	0.005	0.018
Testes	0.003			0.007	0.002	0.010	0.021

on this gland. The comparatively high figures shown by the pituitary gland are, however, slightly deceptive in view of the very small weight of this organ, which is only 10—20 mg. The minute amounts of radio-active sulphur present were only just within the limits of sensitivity of the method. That also the feathers receive a good deal of the given substance is easily accounted for by the fact that the total sulphur content is much higher in the feathers than in the other tissues. It is of interest to note that the activity is concentrated in the bases of the shafts, where the development of the feathers takes place and consequently physiological processes are more intense.

A comparison of columns 1, 2 and 3 shows that after 48 hours the amount of active material present is still considerable and that only after 4 days the activity has almost completely disappeared. By weighing and analyzing the excreta we could ascertain that in 24 hours 60—70 per cent is excreted.

Comparing the first column with columns 4, 5 and 6 we feel we are justified in concluding that there is a definite tendency to cumulation. Especially in the thyroid gland and in the shaftbases is this effect very marked. We have added a few experiments to ascertain, what happens with the last dose. To this end we gave two animals each 9 doses, of which only the last one contained radio-active sulphur. The result, represented in the last column, was rather unexpected: the activity was the same as found in the cases where the animals received 9 active doses. The small number of cases considered so far, makes it difficult to account for this remarkable fact. We intend to continue the investigation on this point.

#### *Acknowledgement.*

We wish to express our thanks to Prof. Dr. A. H. W. Aten Jr. of the Institute for Nuclear Research, Amsterdam, for his kind advice and for his invaluable assistance in the measurements for the radio-activity. The radio-active sulphur was provided by the U. S. Atomic Energy Commission at the request of the Netherlands Isotopes Commission.

#### SUMMARY

The distribution of orally administered methylthiouracil in cockerels was studied by making use of a preparation, containing radioactive sulphur. After 24 hours only the thyroid gland, the shaftbasis of the feathers and probably also the pituitary gland retain appreciable amounts of the drug. The amounts in other organs are insignificant. Excretion has practically come to an end after 96 hours. After repeated dosage there is a definite tendency to cumulation.

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THE INFLUENCE OF PITUITARY GONADOTROPHIN  
AND OF MIXTURES OF PITUITARY AND CHORIONIC  
GONADOTROPHIN ON THE FOLLICLES IN THE OVARY  
OF THE HYPOPHYSECTOMIZED RAT AND  
THE NORMAL MOUSE

BY

F. J. A. PAESI

INTRODUCTION

In a previous paper we analysed the structure of the ovary in the young rat after hypophysectomy and after subsequent administration of chorionic gonadotrophin. We now report the results of a similar analysis in which mice and hypophysectomized rats were stimulated with pituitary gonadotrophin or with a mixture of pituitary and chorionic gonadotrophin.

A number of observations of a more detailed nature have already been made on follicles of different sizes obtained by stimulation of the ovary. Little attention has, however, so far been paid to follicles of the smaller size-groups.

*Lane* (1935) treated young rats with follicle-stimulating hormone (FSH) and observed a 68 per cent increase in the number of follicles with at least two layers of granulosa cells. Smaller follicles were not considered. Treatment with luteinizing hormone (LH) did not affect the total number of follicles,

but the percentage of cavity-containing follicles was markedly increased.

*Guthrie & Jeffers* (1938 a and b) treated bats with pituitary extracts. They found in every case an increase in the number of follicles with a diameter of 24 — 43  $\mu$  and of those with a diameter of more than 164  $\mu$ , but the number of intermediate-sized follicles remained the same. These authors conclude, as *Lane* did, that gonadotrophic hormones may stimulate follicular growth at a very early stage. Our own observations are in agreement with these findings.

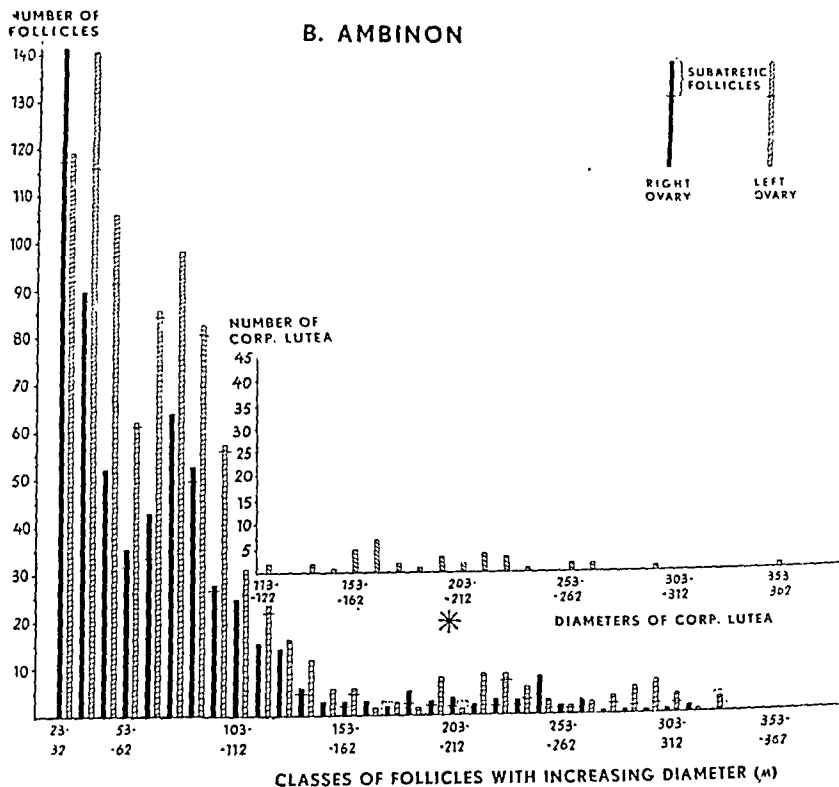
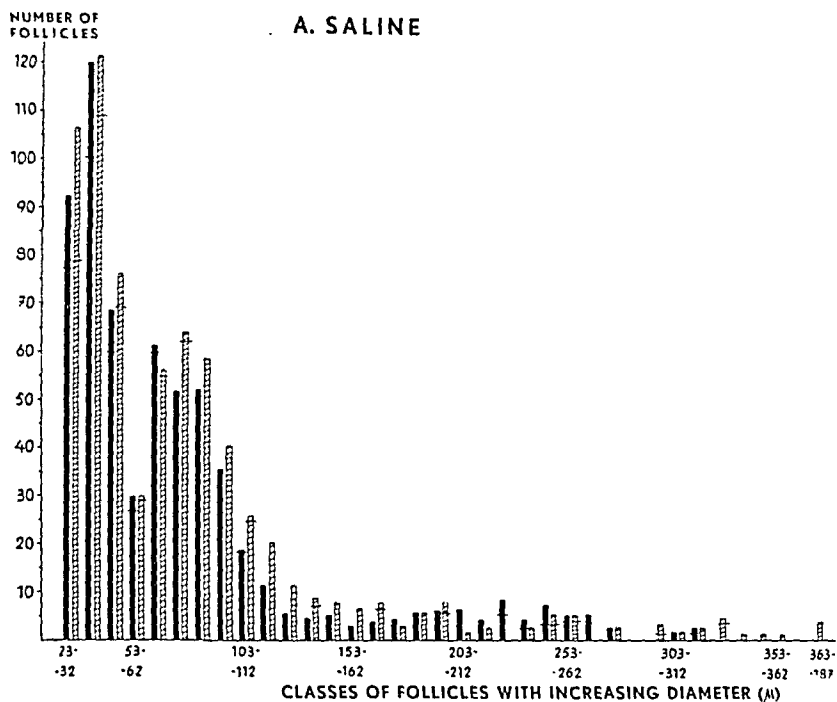
In adult rats during dioestrus *Lane & Davis* (1939) observed an increase in the number of follicles with a diameter between 35 and 100  $\mu$ , which they ascribed to an increase of the mitotic activity in the granulosa during the preceding oestrus and metoestrus; thus the production of oestrogen coincided with the growth of ova and of follicles with a diameter of less than 35  $\mu$ . *Gaarenstroom, de Jongh & Paesi* (1944) found that androgen favours the development of the follicular cavity in normal rats. *Green & Zuckerman* (1947) noticed in monkeys an increase in the number of follicles of different sizes after treatment with androgen; with oestrogen there was a decrease. *Lane & Greep* (1935) administered 'FSH' to *hypophysectomized* young rats, and observed a marked increase in the number of follicles with two or more layers of granulosa cells. *Pencharz* (1940), *Simpson et al.* (1941), *Gaarenstroom* (1942) and *Williams* (1944) found that in *hypophysectomized* rats, *oestrogen* causes small follicles to develop to medium-sized follicles with thriving granulosa, but without cavities.

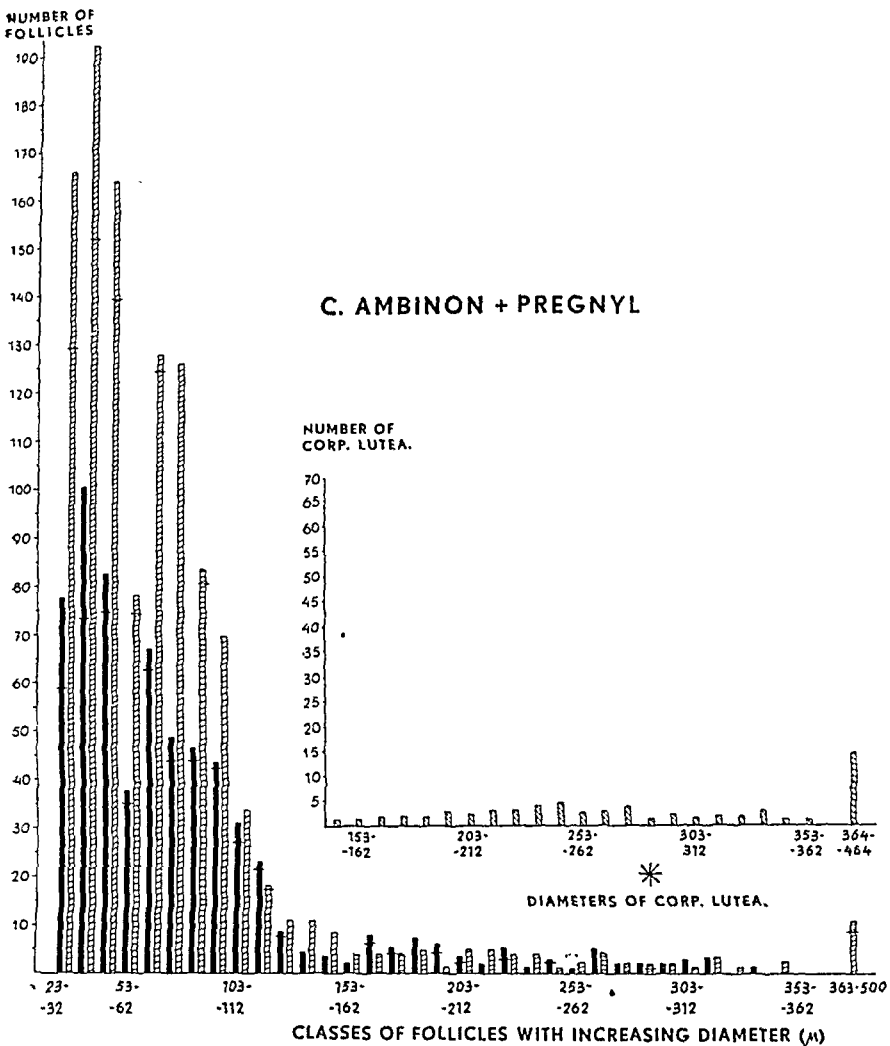
These data suggest that the first stages of the development of the follicle may be stimulated by substances produced by the hypophysis, that oestrogen favours the development of the granulosa and androgen that of the follicular cavity.

## MATERIAL AND METHODS

Our purpose was to investigate further the influence exerted by gonadotrophic hormones on the development of







*Fig. 1.*

Number of follicles and corpora lutea in the ovaries of semi-spayed (r.) young mice, treated during 10 days with: A. Saline, B. Ambinon (2 R. U. daily), C. Ambinon + Pregnyl (2 I. U. daily). Four mice in each group. Left ovary removed on the 8th day.

Black columns: number before treatment (right ovaries).

Shaded columns: number after treatment (left ovaries).

follicles. We therefore injected normal young mice and hypophysectomized young rats with various mixtures of gonadotrophic substances. We measured all follicles (without the theca) and corpora lutea and arranged them in groups according to size (Fig. 1). Our previous paper gives a description of the technique.

Since hypophysectomized young mice could not be kept alive for a sufficient length of time, we used normal mice. Three groups each of four animals (9—14 gm.), were treated over a period of 10 days, respectively with saline, with Ambinon<sup>1)</sup> and with Ambinon and Pregnyl<sup>2)</sup>. The right ovary was removed at the beginning, the left at the end of the treatment. No corpora lutea were present before treatment. Cavity-formation and the first signs of atresia were noted.

In a second experiment hypophysectomized rats were treated with chorionic gonadotrophin for the first eight days following hypophysectomy. From the 9th till the 18th day some of them were treated with pituitary gonadotrophin and chorionic gonadotrophin and some with chorionic gonadotrophin only (Fig. 3).

In the following sections we shall discuss in turn the total number of follicles, the smallest follicles, those with a diameter between 33 and 112  $\mu$ , atresia, the follicles measuring 113  $\mu$  or more, cavity-formation and the corpora lutea. In every section the changes in the hypophysectomized control-animals (A) will be dealt with first; then, those in the Ambinon-treated animals will be discussed (B) and finally those in the animals which received Ambinon and Pregnyl (C).

## RESULTS (mice).

### *The total number of follicles and corpora lutea.*

Figure 2 shows that the hormone treatment caused only minor changes in the distribution of follicles over the various groups.

A. After hypophysectomy the total number of follicles (4 animals) increased from 618 (right ovary) to 686 (left ovary) i. e., by 11 per cent. But the increase was confined to two of the four animals (222—261 and 111—140); in the other two the number remained practically the same (137—131 and 148—

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<sup>1)</sup> Pituitary gonadotrophin, Organon Ltd., at the time rich in 'FSH', dose: 2 R. U. daily.

<sup>2)</sup> Chorionic gonadotrophin, Organon Ltd., dose: 2 I. U. daily.

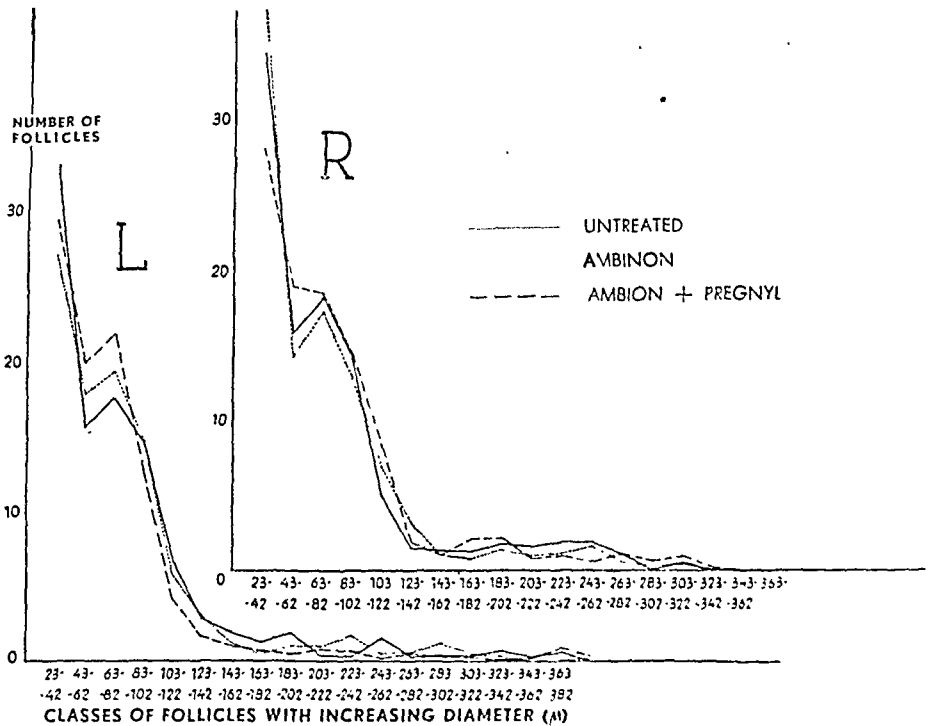


Fig. 2.

Number of follicles in each size-group as a percentage of the total number. In each column two size-groups from Fig. 1 have been combined.

154). This slight increase may be due to the removal of one of the ovaries, causing a 'compensatory hypertrophy' in the remaining one. (Arai, 1920; Engle, 1928). The small changes in the trend of the frequency curve are, moreover, similar to those obtained with gonadotrophic hormones.

B. The total number of follicles plus corpora lutea increased by 55 per cent. Increases in the individual animals: 133—209, 138—285, 80—192, 270—274. The largest follicles were of the same size as found normally. Corpora lutea had been formed.

C. The number of follicles plus corpora lutea increased by 93 per cent. Increases in the individual animals: 93—309, 146—253, 236—446, 154—205. The follicles reached a larger size than in normal circumstances, and there were more and

larger corpora lutea than in the ovaries of animals treated with pituitary extract alone. (B).

*The smallest follicles (23—32  $\mu$ ).*

The number of follicles of this size varies considerably even in animals belonging to the same group. We therefore limit ourselves to the following general statements:

A. The number is slightly increased after removal of the right ovary.

B. There is a slight decrease (142—119) in the animals treated with pituitary extract only, but in view of the high initial value this is of little significance.

C. There is a marked increase in animals treated with the mixture of pituitary extract and chorionic gonadotrophin (77—165).

*Follicles with a diameter of 33—112  $\mu$ .*

The upper limit of this group was fixed at 112  $\mu$  because follicles with a slightly larger diameter may already be luteinizing. This enabled us to compare the corpora lutea with the follicle-categories from which they can be assumed to have originated (see later).

The number of follicles of this size increased in all three groups. In the controls the increase was quite small, but it became much greater under the influence of pituitary extract, and after the joint administration of this extract and chorionic gonadotrophin it reached a still higher value.

Treatment (10 days)	Number of follicles with a diameter 33 - 112 $\mu$ .		Increase in per cent.
	r. ov.	l. ov.	
Saline .....	436	471	8
Ambinon (5 R. U. daily) .....	390	666	71
Ambinon + Pregnyl (5 I. U. daily)	453	870	92

*Atresia.*

Before proceeding to a discussion of the changes in the number of the larger follicles it is convenient to consider the part played by atresia.

The percentage of slightly atretic follicles of all size-groups may be seen in the following data:

*Table 4.*  
Beginning atresia.

Treatment during 10 days with:								
A Saline			B. Ambinon (5 R. U. daily)			C. Ambinon + Pregnyl (5 I. U. daily)		
Mouse Nr.	Percentage of subatretic follicles		Mouse Nr.	Percentage of subatretic follicles		Mouse Nr.	Percentage of subatretic follicles	
	r. ov.	l. ov.		r. ov.	l. ov.		r. ov.	l. ov.
1963	8	10	1899	15	8	1900	16	7
1964	9	10	1907	30	10	1901	7	9
1965	12	10	1908	6	12	1904	11	11
1966	12	7	1909	10	12	1905	19	12
Average: 10				14	11		13	10

The spread makes it impossible to conclude anything more than that the percentage of subatretic follicles shows at most a small tendency to decrease after treatment with gonadotrophic hormones. The same tendency was found in hypophysectomized rats (see later). Should this decrease be significant, then it would be fully explained by the great number of young healthy follicles which were added to the original stock, or by development of corpora lutea from follicles which otherwise would have become atretic (see later). It is therefore unlikely that gonadotrophic hormones grossly diminish the tendency of the follicle to degenerate, i. e., lengthen their 'life'. In our previous paper we came to the same conclusion. Fig. 1 shows that the largest percentage of atretic follicles is always found among the smallest and the largest follicles. Administration of gonadotrophic hormones leads to a further decrease of the

atresia among the follicles of medium size, and causes a shifting of this process towards the largest ones. This shift is much more conspicuous in C than in B. Addition of chorionic gonadotrophin to the pituitary extract causes atresia to disappear completely from the follicles measuring 123—282  $\mu$  and to be observed only in follicles larger than 282  $\mu$ .

*Follicles with a diameter of 113  $\mu$  and more.*

Follicles of this size did not always increase in number during hormone treatment; in several groups a decrease was even noted. In Table 2 this group is divided into one in which the diameter varies from 113 to 322  $\mu$  and another in which the larger follicles are brought together.

Table 2.  
Follicles larger than 112  $\mu$  and corpora lutea.

	A. Solvent		B. Ambinon (5 R. U. daily)		C. Ambinon + Pregnyl (5 R. U. and 5 I. U. daily)	
	r. ov.	l. ov.	r. ov.	l. ov.	r. ov.	l. ov.
Number of follicles with diameter of 113—322 $\mu$ .....	91	97	89	134	98	99
(In brackets the number of atretic ones) .....	(13)	(11)	(15)	(27)	(12)	(2)
Number of follicles with diameter larger than 322 $\mu$ ....	0	11	0	3	1	14
Corpora lutea (+ partially luteinized foll.) .....	0	0	0	39	0	65
Total numbers (follicles + corpora lutea) .....	91	108	89	176	99	178

In the left ovaries of the control-animals the largest follicles became larger. This also happens when gonadotrophic hormone is administered (which will have been inherent in the compensatory hypertrophy in the control-group).

The increase in the number of follicles larger than 112  $\mu$  (plus c. lutea) was approximately the same in B and C. There were, however, more follicles up to 323  $\mu$  in B, than in C,

whereas a greater number of follicles *larger* than 322  $\mu$  and corpora lutea developed in C. Thus, additional chorionic gonadotrophin caused a number of follicles doomed to become atretic when of medium age to develop into large follicles or corpora lutea. This shift is illustrated in Fig. 2L; the part of the curve representing the follicles measuring 182—342  $\mu$  is found constantly higher in B than in C, and this was not the case before treatment (Fig. 2R). Above 342  $\mu$  the situation appears to be reversed. The shift of atresia towards the largest follicles agrees with this theory.

### *Cavity-formation.*

The percentage of cavity-containing follicles was determined in those groups in which a cavity first became noticeable (105—212  $\mu$ ). Neither in A nor in B could any change in the percentage of developing cavities be noted (A: 42—42; B: 33—31). In C, it decreased (75—54); it is difficult, however, to evaluate the significance of this decrease since the initial value was exceptionally high. Curiously enough Lane (1935) observed a marked *increase* in the percentage of cavity-containing follicles when pituitary 'LH' was administered to normal animals.

### *The corpora lutea.*

The data on the corpora lutea are best seen in the small graphs given in Fig. 1. Since no corpora lutea in *advanced* stages of degeneration were observed the number of counted corpora lutea was considered to represent the total number formed during treatment. The asterisk (\*) indicates the average diameter in  $\mu$ . The principal points are:

1) Pituitary extract plus chorionic gonadotrophin (C) causes more corpora lutea to develop than pituitary extract alone (B).

2) The corpora lutea in C are on the average larger than those in B.

3) In C the corpora lutea show a lesser tendency to degenerate than in B; in B, 26 among 39 were degenerating, in C: 11 among 65.



4) The diameter of the largest corpora lutea never exceeds that of the largest follicles. The majority measure only 150—250  $\mu$ .

Since degenerating corpora lutea become smaller, one might feel inclined to assume a relation between points (2) and (3). However, the difference in size between normal and degenerating corpora lutea is only a fraction of that between the corpora lutea in the two hormone-treated groups; this is shown in the following table:

Average diameter of corpora lutea.

	A. (Controls)	B. (treated with Ambinon)	C. (treated with Ambinon + Pregnyl)
»Healthy« corpora lutea ..	none	195 $\mu$	288 $\mu$
Degenerating corp. lut. ...	none	185 $\mu$	278 $\mu$

An explanation must therefore be sought in the way in which the corpora lutea are formed: in group C larger follicles are to be found than in B, and thus, it is not surprising that C also contains the largest corpora lutea. Further increase in size during luteinization is not likely to occur, the largest corpora lutea being of about the same size as the largest follicles.

There proved to be no relation whatever between the percentage of cavities or the increase in the number of follicles on the one hand and the number of corpora lutea formed in the individual animals on the other hand.

## RESULTS (rats).

### *The joint effect of pituitary and chorionic gonadotrophin on the ovary of the hypophysectomized rat.*

Three young rats were hypophysectomized, and during the period from the 9th to the 18th day after the operation they were injected with Ambinon (5 R. U. daily) and Pregnyl (5 I. U. daily). Other hypophysectomized rats were injected with Pregnyl only; these were intended as controls but with a single

exception they all died prematurely. All animals received Pregnyl during the first week. This was used for a study of the interstitial tissue which will be described elsewhere (*Paesi*, 1949).

The changes brought about in the ovaries of hypophysectomized rats by Ambinon and Pregnyl proved to be of the same nature as those in normal mice:

(1) a marked increase in the number of follicles from the smallest primordial follicles up to those with a diameter of ca. 100  $\mu$ ;

(2) a *decrease* in the number of follicles in many size-groups above 100  $\mu$ ;

(3) larger follicles than would have been produced under ordinary circumstances, and the presence of many corpora lutea;

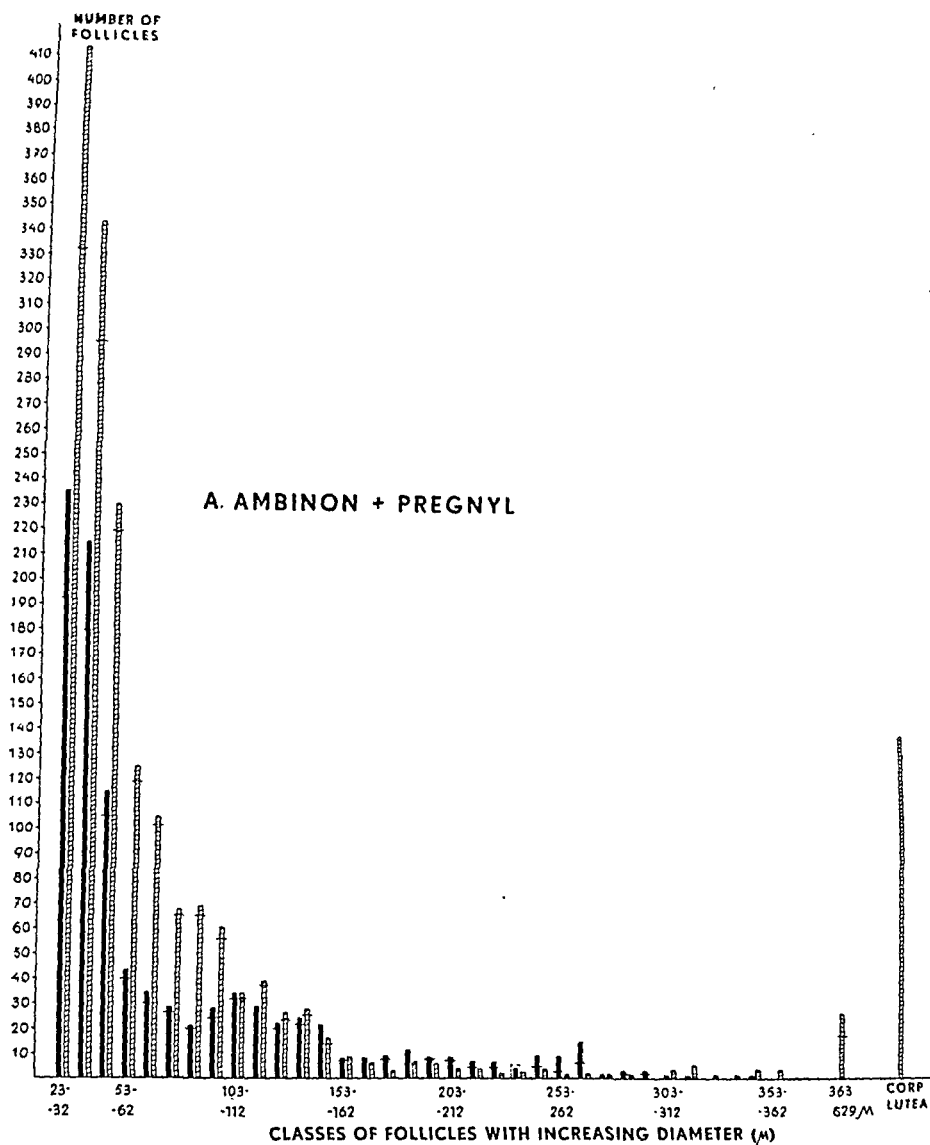
(4) a decrease in the number of slightly atretic follicles from 14 to 11 per cent, and a shifting of the enhanced atresia of larger follicles towards groups with a still larger diameter; these began at 213  $\mu$  before treatment, and reached 366  $\mu$  after treatment.

There was, moreover, a slight decrease in the percentage of cavity-containing follicles in those size-groups in which cavities first made their appearance (93—212  $\mu$ ), i. e., from 66 to 48 per cent.

The rat that survived treatment with chorionic gonadotrophin alone for 18 days had become refractory to this substance (*Paesi*, 1949). Because of this change in sensitivity a comparison of both ovaries gives no idea of the normal effect of chorionic gonadotrophin after hypophysectomy (see our previous communication), but showed the opposite, i. e., the disappearance of this effect:

(1) on the 18th day the interstitial tissue was found to be atrophic whereas it was still hypertrophied in the ovary removed on the 8th day;

(2) between the 8th and the 18th day the number of the smallest follicles (diameter 23—42  $\mu$ ) showed a marked increase, just as it would have done without any treatment



during the *first* week after hypophysectomy. Chorionic gonadotrophin would however, have *interfered* with the increase during this period.

## DISCUSSION

In a previous paper we pointed out that the development of primordial ova into primordial follicles of the smallest size-

## B. PREGNYL

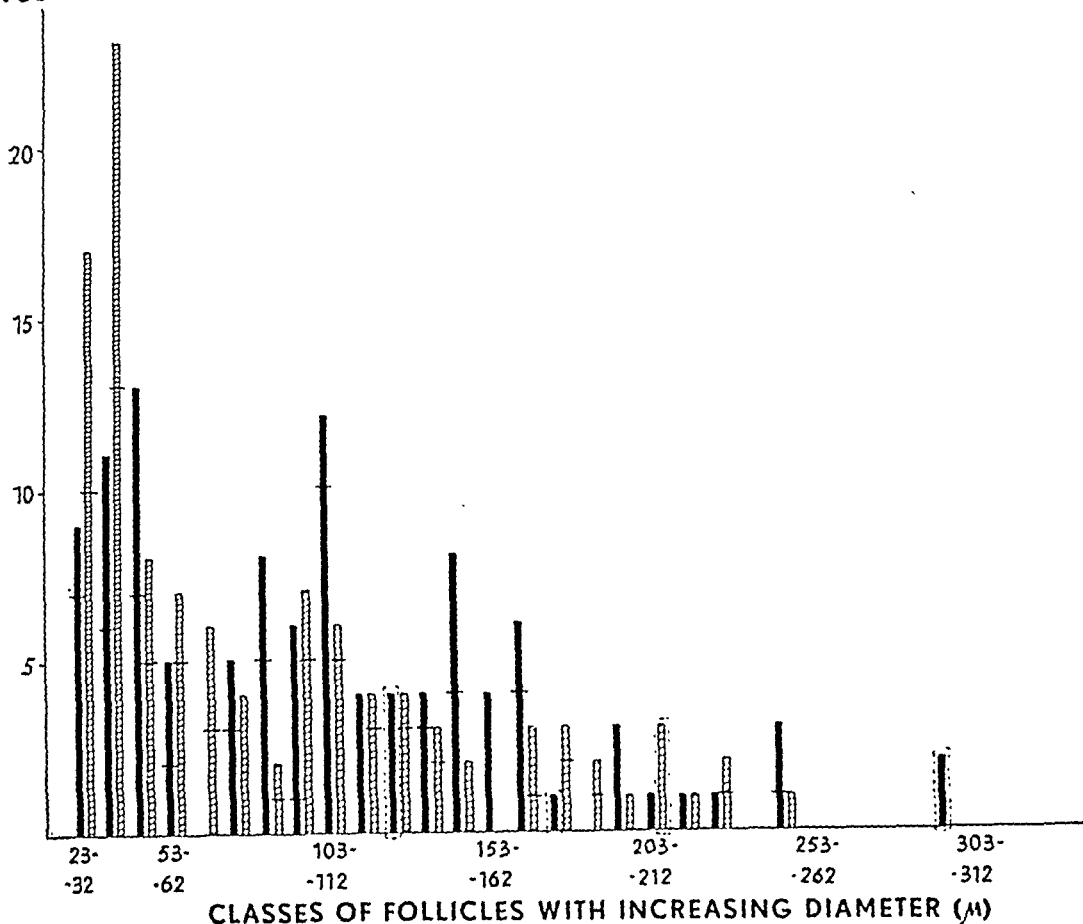
NUMBER OF  
FOLLICLES

Fig. 3.

Number of follicles in the ovaries of young hypophysectomized rats. Treatment: Pregnyl (5 I. U. daily) during 7 days following hypophysectomy; removal of right ovary on the 8th day (black columns); further treatment with Pregnyl + Ambinon (5 R. U. daily): 3 animals, or with Pregnyl only: 1 animal, during 10 days; removal of left ovary (shaded columns).

group is less dependant on the presence of the hypophysis than are the subsequent stages of the follicular development. We were now able to demonstrate that the follicles of the smallest size may also show a marked increase in number under the influence of gonadotrophic hormone. Therefore, although the

development of the first layer of granulosa cells is impaired comparatively little by hypophysectomy we must conclude that it can nevertheless be considerably stimulated by an adequate supply of gonadotrophin.

The development of the primordial into larger follicles is depressed by hypophysectomy. In agreement with this result gonadotrophic hormones appeared to stimulate this development.

Stimulation may manifest itself in various ways: The 'life-time' of the follicles may be lengthened, their number or their growth rate increased, their 'maturation' (including luteinization) stimulated.

Evidence derived from data on atresia suggests that there is no marked lengthening of the period of life of the follicle.

The number of follicles was increased by pituitary gonadotrophin. A greater increase was obtained, if chorionic gonadotrophin was also administered (group C), but this additional gain was confined to follicles up to ca. 110  $\mu$ . Thus, those extra-follicles developing in C evidently did not reach larger diameters than 110  $\mu$  by the time the experiment was terminated.

There is good evidence that at least the growth rate of the medium-sized follicles was increased in C. In a preceding section we explained that the large average diameter of the corpora lutea in C must be due to the large diameter of those follicles which were about to luteinize. Of the two types of changes which may lead to development of follicles of such diameters: acceleration of growth and decreased tendency to luteinize, only the first one can be considered, since of the follicles larger than 110  $\mu$  a greater percentage developed into corpora lutea if chorionic gonadotrophin was administered. This speeding up of growth coincides with cavity-formation and may, at least partly, have been due to the enlargement of preformed cavities.

We did not pay any attention to maturation phenomena such as extrusion of the polar bodies and the possible effects of

gonadotrophins on it (*Moricard*, 1940). They were beyond the scope of this investigation.

Besides the increase in number and size of the corpora lutea in C as compared to B their trophic condition was maintained for a longer period in group C than in B. Similar effects are obtained when, instead of chorionic gonadotrophin, oestrogen is added to the pituitary extract (*Fevold & Hisaw*, 1934; *Selye & Collip*, 1936; *Robson*, 1937; *Freud & Dingemanse*, 1941). As oestrogen production is stimulated by chorionic gonadotrophin in the presence of pituitary extract (*Collip et al.*, 1933, *Evans et al.*, 1934; *Leonard & Smith*, 1934; *Fevold*, 1941), it is possible that oestrogen was responsible for several of the effects mentioned. Since, however, vaginal cornification was absent in all our animals, any amount of oestrogen produced must have been very small indeed.

In a subsequent paper we shall attempt to establish a relation between the shape of the curve representing the number of follicles and their rate of growth.

### SUMMARY

The influence of gonadotrophic hormones on the ovary was studied in experiments on young mice and on young hypophysectomized rats. One ovary was removed before and the other at the end of treatment. All follicles and corpora lutea were measured and arranged in groups according to their size.

In normal mice, pituitary extract induced the formation of corpora lutea and increased the total amount of follicles and corpora lutea by 55 per cent. Addition of chorionic gonadotrophin to the extract raised this increase to 93 per cent but this additional increase was limited to the follicles with a diameter up to ca. 110  $\mu$ . The number of corpora lutea produced during the joint administration of pituitary and chorionic gonadotrophin was nevertheless larger than the number produced by pituitary extract alone (respr. 65 and 39). They were, moreover, larger on the average (although never exceeding the largest follicles) and showed less tendency to atrophy. The

addition of chorionic gonadotrophin was shown to be followed by an acceleration of the growth rate of medium-sized follicles.

Similar results were obtained in hypophysectomized rats. For several reasons, however, these were less conclusive than those obtained in mice.

The percentage of cavity-containing follicles was in no case increased by the treatment. Atresia showed no conspicuous change.

In normal mice, removal of one of the ovaries always led to a slight increase in the number of follicles in the remaining ovary, nearly all size-groups contributing to this increase.

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THE RELATION BETWEEN THE RATE OF GROWTH  
OF THE OVARIAN FOLLICLES AND THE SHAPE OF THE  
FREQUENCY CURVE REPRESENTING THEIR  
VARIABILITY IN SIZE

BY

F. J. A. PAESI

In two previous communications (*Paesi*, 1949 a and b) figures were given of the number of follicles of various size found in the ovaries of rats and mice. It was noteworthy that the curves based on these figures always showed some common features, no matter whether the animals had been hypophysectomized or treated with various gonadotrophins. They conformed to a definite type with asymptotic approach to the abscissa axis, but with certain constantly-recurring deviations from the ideal shape. This curve is shown in fig. 1.

The two main questions to be answered are: To what is the 'logarithmic' form of the follicle curve due? What causes the main deviations from the ideal shape?

The general resemblance of the follicle-curve to an asymptote might be explained in one of several ways:

1) Since in each subsequent stage of development a number of follicles will be eliminated on account of atresia, the decrease in the number of follicles found in the successive size-groups might be due to this factor. Indeed, if the *percentage* of atretic follicles found in the various size-groups were about



the same, the trend of the curve would be a logarithmic one. However, this percentage shows in the successive groups well-marked and quite typical fluctuations and in follicles of a

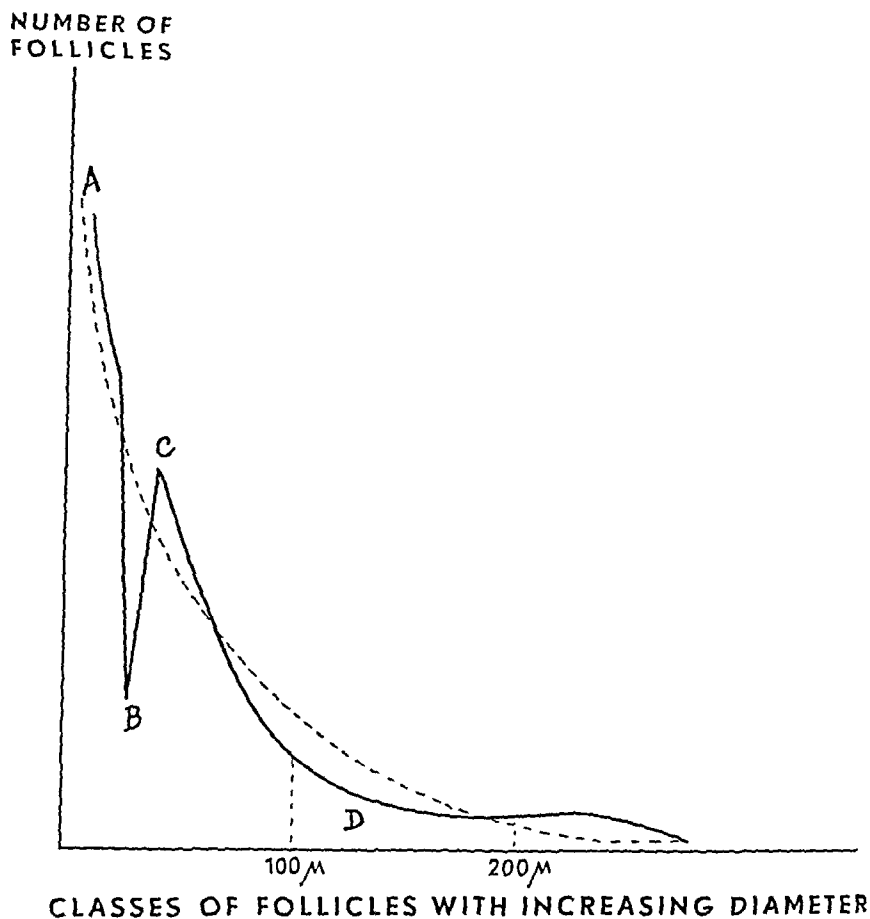


Fig. 1.

Schematic course of curves representing the number of follicles of different size, when divided into size-groups according to an arithmetically increasing diameter. Dotted line: principal trend.

diameter between  $83\ \mu$  and  $142\ \mu$  there is practically no atresia at all, even though in these groups there is a marked decrease in number. In some parts of the graph, moreover, the numbers increase, i. e., the curve rises. This too is in contradiction to the atresia hypothesis. Such an increase can only be due to a

change in the *rate of growth*, and our second explanation is based on the occurrence of changes of this kind.

2) The 'linear' growth rate of the follicle might increase steadily. This would mean that the time a follicle requires to increase its diameter by  $10\ \mu$ , i. e., the time required to pass to the next higher group, is gradually shortened. *The chance of finding follicles, belonging to the same size-group as a follicle selected at random is therefore smaller, the larger the follicle selected.*

Evidence for this theory is furnished by the work of Lane & Davis (1939). They counted the number of mitoses in the granulosa of follicles found in the ovaries of rats, and calculated from the actual data the number that would have been present in 0.001 cubic millimeter of granulosa tissue; this they called the 'mitotic index'. Assuming the average size of the granulosa cells to remain approximately constant, this means that in follicles with a diameter up to  $200\ \mu$ , the *volume of the granulosa increases per unit of time on the average by nearly a constant* — or at most a slightly increasing — *percentage*.

A decrease in the average size of the cells might simulate an increase in the rate of growth even when the latter had in fact undergone a decrease, because it would have led to an increase in the number of cells in the given area where the mitoses were counted! In our ovaries, however, no indication of such a decrease in size was found. As long as the development of the cavity can be left out of consideration, i. e., in follicles with a diameter of less than  $100\text{--}150\ \mu$ , and assuming, that the egg cell grows up to then at the same rate as the granulosa, as it probably does (see below), we may replace the volume of the granulosa by that of the follicle.

When the volume of a spherical body increases in geometrical progression, its *diameter* increases in each subsequent unit of time by a larger amount than in the preceding one. In other words: the time required to obtain an arithmetical increase in diameter (in the case of our follicles :  $10\ \mu$ ) will gradually decrease. Thus, the larger the sphere (follicle) with geometrically progressing *volume* grows, the smaller the chance to find it in a size-group covering a definite increase in *diameter* ( $10\ \mu$ ). In fact, the number of spheres counted in

such size-groups will vary in inverse proportion to their mean diameter; this is shown in the 3rd and 4th lines of Table 1. When a graph is made of the number of such expanding spheres that will be present in size-groups differing by a fixed amount of length-units it will assume the shape of an asymptote (see fig. 2, broken line).

When the spheres increase e. g. in the unit of time by twice their volume, we obtain the results shown in Table 1:

Table 1.

Interrelation between the growth of spheres with geometrically progressing *volume* and their number counted in size-groups representing a definite (i. e., arithmetical) increase in *diameter* per unit of time.

1. Volume of expanding sphere in units of time, expressed in an arbitrary space unit.	10	30	90	270	810	2430	7290	21870	65610
2. Cube-root of this volume, i.e., diameter.	2,1	3,1	4,5	6,5	9,3	13,4	19,4	27,9	40,3
3. Increase in diameter (a) in successive units of time.	1.0	1.4	2.0	2.8	4.1	6.0	8.5	12.4	
4. Number of spheres $\left(\frac{100}{a}\right)$ in size-groups differing by a fixed number of units of length.	100	71	50	36	24	17	12	8	

The observations of *Lane & Davis* are, thus, in good agreement with our own findings. They have, however, not yet been confirmed by other investigators, nor do we know whether the

number of mitoses per unit of volume varies when different kinds of stimuli are applied.

There are some striking deviations from the ideal shape of the curve. We will discuss these under two headings:

*a.* In the first place we find a 'depression' between B and C. These changes must have been caused by an acceleration followed by a retardation of the follicle-growth due to changes in the rate of growth either of the ovum, or of the granulosa, or of both.

Preliminary measurements led to the conclusion that the ovum does not show at any stage of its development a faster growth than the follicle as a whole (i. e., than the granulosa).

In the mouse an examination of the granulosa at the time at which the depression reaches its lowest value, revealed the presence of a single layer of particularly high cells with nuclei shifted towards the periphery. During the next phase of their development these large cells divide transversely and the granulosa becomes two-layered. We must assume that the increase in height which precedes the division takes place within a relatively short time whereas the increase in size after the division is a comparatively slow process. The ovum too will possibly grow faster during the first period; *Moricard* (1940) describes a relation between the metabolism of the ovum and the 'palisade layer'.

When comparing the follicles of rats and mice, it seemed to us that the phase during which the granulosa cells of the first layer reach their full height (i. e., the phase which precedes the division) is in the rat less sharply separated from the next stage than it is in the mouse. In the rat the development of the second layer of granulosa cells is more gradual; this would explain why in the rat the 'depression' is spread over a greater number of groups than in the mouse, but does not reach the same depth. The differences which such fluctuations in the rate of growth would cause in the shape of the graphs, are shown in Table 2 and in the three graphs of Fig. 2.

Table 2.

Influence of accelerations and retardations of the growth rate of spheres with *geometrically* progressing volume on the trend of curves representing their number in *arithmetically* increasing size-groups.

Volume of expanding sphere in successive units of time, expressed in an arbitrary space unit.	10	30	90	270	810	2430	7290	21870	65610
						4000	12000	36000	108000
							(8860)	(23440)	(67180)
Cube-root of this volume, i.e., diameter	2.1	3.1	4.5	6.5	9.3	13.4	19.4	27.9	40.3
						15.9	22.9	33.0	47.7
							(20.7)	(28.6)	(40.7)
Increase in diameter (a) in successive units of time.	1.0	1.4	2.0	2.8	4.1	6.0	8.5	12.4	
					6.6	7.0	10.1	14.7	
						(4.8)	(7.9)	(12.1)	
Number of spheres ( $\frac{100}{a}$ ) in size-groups differing by a fixed number of units of length.	100	71	50	36	24	17	12	8	
					15	14	10	7	
						(21)	(13)	(8)	
In Fig. 2:	A	B	C	D	E	F	G	H	

If an acceleration of growth is supposed to set in when a volume of 810 has been reached, the sphere will expand in the next unit of time e. g. to a volume of 4000 instead of 2430. If the original growth rate is presently resumed, the sphere will be found to expand to  $3 \times 4000 = 12000$ , then to 36000, 108000, etc., in the ensuing time-units. The corresponding numbers in the fourth line (italics) show clearly that an acceleration of growth causes a fall in the frequency curve which, however, is not followed by a rise (see the broken line in Fig. 2).

To explain the subsequent rise which we observed in our follicle-curves, we must assume that follicle-growth does not return to its *former* rate immediately after having been accel-

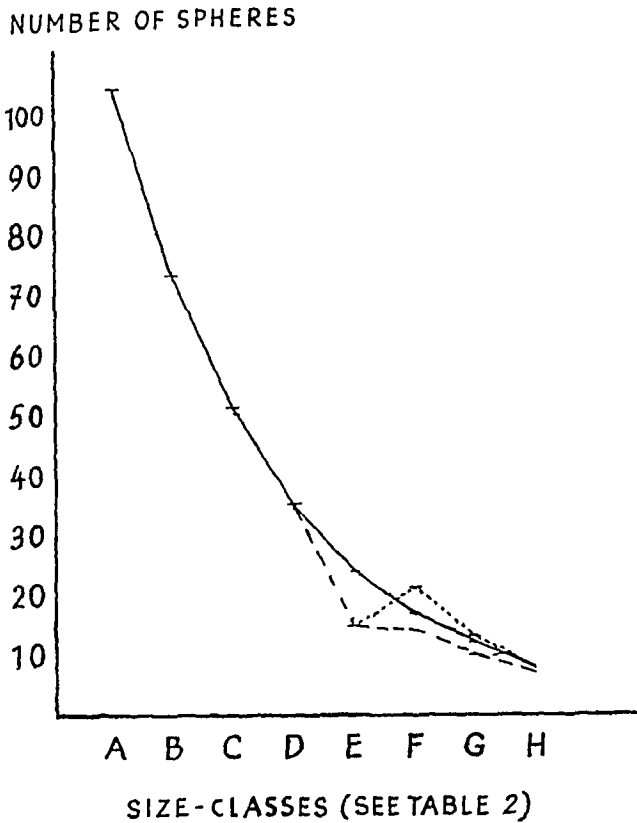


Fig. 2.

Number of expanding spheres with volumes increasing in geometrical progression, when divided into size-groups according to an arithmetically increasing diameter (compare Table 2).

- graph representing the case in which the rate of growth remains strictly constant (based on figures calculated for an increase of 200 per cent per unit of time).
- - - - - The rate of growth is temporarily accelerated; at E the original rate of growth is restored.
- ..... The rate of growth is accelerated, and at E retarded to a lower rate than the original one.

erated, but to a *slower* rate. We may for instance assume that the growth rate is slowed to a rate which allows the volume to increase per unit of time by the *absolute* differences between

the volumes attained should the growth have proceeded undisturbed (e. g.: 7290 - 2430, 21870 - 7290, 65610 - 21870).

The numbers in brackets (Table 2) and the corresponding dotted line in Fig. 2 make it clear that in this way depressions followed by peaks are obtained which resemble the depressions observed in our follicle-curves.

b. In all our follicle-curves for both rats and mice a second fall is found at the point marked D, between ca. 100 and 150  $\mu$  (cf. Fig. 1). It is followed by a slight rise which produces a peak at a diameter of 220—250  $\mu$ . These changes may be due to the formations of a third layer of granulosa cells. This layer is formed more gradually than the second, thus causing a longer lasting and shallower depression. The development of the cavity, which begins when the follicle has reached a diameter of about 100  $\mu$ , may well cause an additional increase in the rate of growth.

## SUMMARY

The general trend of the graph representing the number of ovarian follicles of different size becomes comprehensible if we assume that the volume of the follicle increases — at least before cavity-formation sets in — by a nearly constant percentage in each unit of time.

For some constantly-recurring deviations from the ideal shape an explanation was sought in temporary changes in the rate of growth.

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## ANEMIA AND ARTHRITIS IN A CASE OF PITUITARY INSUFFICIENCY CONFIRMED AT AUTOPSY

BY

H. HORTLING

Pituitary insufficiency or Simmonds' disease is found in women more often than in men. The most common cause of this disorder is necrosis of the hypophysis, as a rule due to hemorrhages associated with partus or septic emboli. Less common causes are inflammatory processes of tuberculous or syphilitic origin, and tumours of different kinds. The general symptoms, mentioned in order of frequency, are: diminished sexual function, low basal metabolic rate, loss of hair, cachexia, lowered insulin tolerance, achylia, progeria, atrophy of the mammary glands, anemia, eosinophilia, subnormal temperature and skin pigmentation (*Sheehan*, 1939, *Escamilla & Lissner*, 1942, and others). In the following, a case of pituitary insufficiency due to pituitary fibrosis is reported in a male which showed, besides the classical symptoms of Simmonds' disease, an arthritis with a raised blood sedimentation rate and an anemia of aplastic type.

*History.* The patient was a sailor, born in 1893. Heredity non-contributory. Cannot recollect any diseases in childhood. Three times gonorrhoea before 1928. Denies syphilis.

In the spring of 1928 the patient was working on a ship sailing on the Atlantic. The journey was difficult and afterwards he felt ill



and tired. He was admitted to hospital at Le Havre, where he lay for a month with fever but without any other noteworthy symptoms. He was removed to hospital at Hamburg. The fever persisted for a further three weeks and the patient was finally discharged after three months in hospital. From time to time he had intestinal bleeding and vomiting, but no abdominal pain. He received large injections in the thighs. As a result of the diseases the hair on the whole of the body fell off. The blood sedimentation rate in December 1928 was 97 mm./1 hour. Ever since this illness the patient felt tired and out of sorts. The right leg was somewhat stiff and it was therefore treated with massage. He regularly visited the dispensary of Maria Hospital, Helsingfors, where a constant hypochromic anemia of moderate and varying degree was recorded. He was admitted seven times to the medical department of the same hospital. Gradually he lost weight and vigour and became unfit for regular work. He was always cold, sometimes had pains in his legs but no fever, and no pains in his joints. The hair on the head and trunk generally returned in a few years but the pubic and axillary hair remained very scanty. The skin was dry. After his illness in 1928 the testes were small and soft and from 1942 he was impotent.

In the summer 1945 the patient was rather better and he even helped with the reaping. He had no special pains nor stiffness in the joints. In the middle of October he fell and hurt his left thigh. At the surgical dispensary of the above mentioned hospital no fracture could be detected. As the leg, however, remained stiff and aching the patient was again admitted on November 16, 1945 and since then he never left the hospital. Gradually the pain in his left leg increased, stiffness in the knee followed and the muscles and tendons of the leg were stretched and sore. In August 1946 the left leg could no longer be straightened. The left knee felt warmer than the right one. The sedimentation rate, which had always been high, showed even higher values and the anemia increased in spite of treatment. As a consequence of an attempt to correct the left knee in the surgical department by immobilizing the leg after straightening it by force, his already weak general condition got worse. Death occurred on October 10, 1947 at 54 years of age.

*State.* In the following a detailed report is given of the state of the different organs as examined during the patient's admissions to hospital. The hospital diagnoses were: 1929 Encephalitis chronica?; 1933 Infectio acuta, Anaemia secundaria, (Hypogenitalismus); 1941 Anaemia secundaria, Dysendocrinismus; 1944 Dysendocrinismus, Achylia gastrica, Anaemia secundaria; 1945 Dysendocrinismus Anaemia secundaria.

The patient was of slender constitution, weight 1929: 52 kg., 1934: 59 kg. and after that gradually declining, in November 1945: 48 kg., in July 1947: 38 kg. Height 165 cm. He looked more than his age, his face was wrinkled, complexion yellow pale with small dirty-brown stains. Mucous membranes without pigmentations. Sclerae not icteric. No enlarged lymph nodes. No edema. *Thyroid gland* palpable, small, containing no nodules. Metabolic rate at five determinations during the years 1941—1947: 21, 24, 29, 26, and 9 per cent below zero. Serum cholesterol 1946 290 mg. per cent. Repeated administration of thyroid preparation in large doses did not affect the patient's general condition nor his anemia. Thyrotrophic hormone was tried in the form of the preparation Ambinon Organon but without apparent effect. — Serum calcium 1945 11.6 mg. per cent (10.9—12.4). *Respiratory organs.* Lungs normal except that X-ray examination revealed a sharply limited spot, the size of a finger tip, in the left infraclavicular region. No tubercle bacilli were found in the sputum. *Circulation organs.* Pulse normal. Blood pressure 1929 was 80 mm. Hg, 1939 90 mm. Hg and subsequently between 110/60 and 145/75 mm. Hg. After subcutaneous injection of 0.7 ml. 1 per mille adrenaline solution the blood pressure showed no rise 5, 10, 15, 30 and 60 minutes after the injection. On the contrary it rather showed a tendency to fall (125/80—100/55 mm. Hg) while the blood sugar as well as pulse rate increased. An increase of the neutrophil granulocyte and the lymphocyte counts within one hour after the adrenaline injection was also noted. An X-ray revealed cor parvum. Electrocardiogram 1946: Right axis deviation, P small, hardly detectable, PQ O. 15, T 1—2 positive, T3 negative, small deviations, rhythm regular. No clinical symptoms of heart disease. *Digestive system.* Liver and spleen not enlarged. At four Ewald's test meals free hydrochloric acid was absent. X-ray of the ventricle revealed nothing pathological except ptosis. The patient sometimes complained of mild dyspeptic troubles. Meulengracht's icterus index in the years 1943, 1946 and 1947, 1:7, 1:4 and 1:2, respectively. In the urine, Schesinger's test (urobilin) was negative in 1945 and 1947. In the stool there were no worm-eggs and no evidence of blood. Takata's reaction negative 1946. *Carbohydrate metabolism.* Adrenaline test (0.7 ml. Exadrin Astra intramuscularly): Blood sugar level increased from 0.061 mg. per cent before injection to 0.113 30 minutes later and 0.065 after 2½ hours. Glucose tolerance test on March 3, 1945: blood sugar rose from 0.071—0.127 (after ½ hour) — 0.103 (1 hour) — 0.080 (1½ hours) — 0.065 (2½ hours); April 6, 1945: blood sugar rose from 0.063—0.099 (½ hour) — 0.096 (1 hour) — 0.054 (2½ hours). The rise in blood sugar was less than normal, a fact which can easily be correlated with a disturbance of the function of the anterior lobe of the hypophysis. Insulin tolerance



roid preparations, Doca and pituitary anterior lobe preparation had no apparent effect on the joint symptoms (regarding doses see later). It is remarkable that during the various admissions to hospital the patient had fever only very occasionally in spite of the raised blood sedimentation rate. The values of the blood sedimentation rate during the first hour varied in 1933 between 37 and 70, in 1941 between 81 and 104, in 1943 between 65 and 95, in 1945 between 29 and 120, in 1946 between 104 and 141, in 1947 between 71 and 157 mm. on Oct. 17. It should be noted that the patient had a markedly raised blood sedimentation rate long before his joint troubles developed. During his admissions to hospital no trouble of this kind worth mentioning in the diagnosis occurred. X-ray examination of the thoracic and sacrolumbar vertebrae and the left knee in the years 1946—47 revealed a small arthrotic point in one of the lower thoracic vertebrae and atrophy in the bone of the left knee. The formolgel reaction was negative in the serum, but positive 45 minutes in plasma in December 1945. Serum proteins determined in the years 1946—47 varied between 5.9 and 7.7 gm. per cent while the globulin at three determination (Biuret method) varied between 3.1 and 4.27. The alkali reserve was 44.8 vol. per cent.

*Hematopoiesis.* Ever since the patient was admitted to Maria Hospital for the first time the presence of a normochromic anemia was recorded; it gradually increased and on Sep. 24, 1946 reached the lowest count: hemoglobin 35/41 per cent Sahli, erythrocytes 1.970 millions per cmm., colour index 1.04 (see the diagrams). The erythrocyte diameter was 7.57 and 7.58  $\mu$  when measured in 1945 and 1946. As previously mentioned, Meulengracht's icterus index was normal and the urobilinogen reaction in the urine was negative. The reticulocyte values usually remained below 1 per cent (25 determinations). As a result of treatment small increases in reticulocytes occasionally occurred (maximum 3.4 per cent). Thus there was no evidence of blood destruction, but it should be mentioned that the blood cell resistance was not determined. Hematocrit 21 vol. per cent at two different determinations June 26 and July 16, 1947. Serum iron at two determinations 1946 0.102 and 0.114 mg. per cent. According to L. E. Tötterman, who was kind enough to undertake the determinations, these values fall within the limits of the normal variation. The leucocyte count showed a tendency towards depression and in the last years clear leucopenia appeared. In 1929 the average of 3 determinations was 7300/cmm., ranging from 5000—8500, in 1933 the corresponding figures were 4, 5420, 4600—6180; 1941: 8, 4400, 3100—5300; 1943: 1, 3700; 1945: 6, 4300, 3600—5000; 1946: 46, 3600, 2100—5700; 1947: 12, 3700, 2400—5000. The differential count showed

nothing pathological except a slight tendency to relative lymphocytosis. Eosinophilia was not proved. The leucocytes looked normal. The thrombocyte count also showed a tendency towards low values. The average of 12 determinations in 1946 was 81702/cmm. (only one value exceeded 100.000, namely 307.972; the lowest value was 31000) and of 17 determinations in 1947 the mean value was 84057 (5 determinations gave values above 100.000; the lowest value was 39000/cmm.). The bone marrow was examined in 1941, 1945 and 1946. The first two times the bone marrow picture resembled the peripheral blood picture. This fact appears to indicate a strong addition of blood. The test in 1946 gave a specimen with few cells and single normoblasts, myelocytes and metamyelocytes and otherwise rather resembling the peripheral blood picture. The presence of anemia,

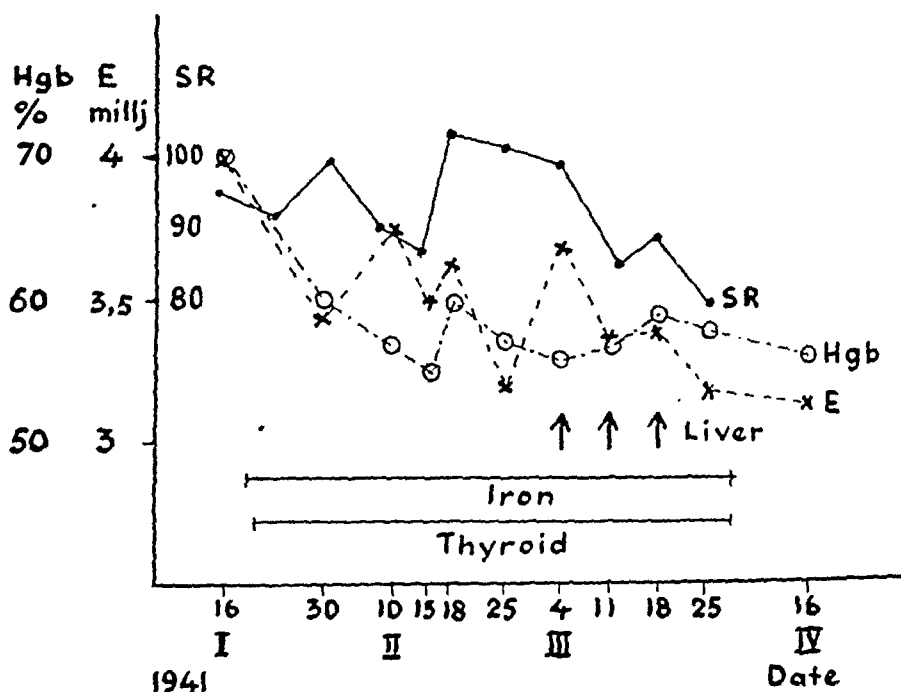


Fig. 1.

Blood sedimentation rate (SR), hemoglobin (Hgb) and erythrocytes (E) during iron, thyroid and liver therapy.

leucopenia and thrombocytopenia without macrocytosis or raised colour index appears to indicate an early aplastic anemia. The therapeutical results are partly described in the diagrams, where hemoglobin (Hgb), erythrocytes Er) and in some cases reticulocytes and the blood sedimentation rate (SR) have been indicated (Figs. 1—2). As partly demonstrated by the diagrams, no apparent effect

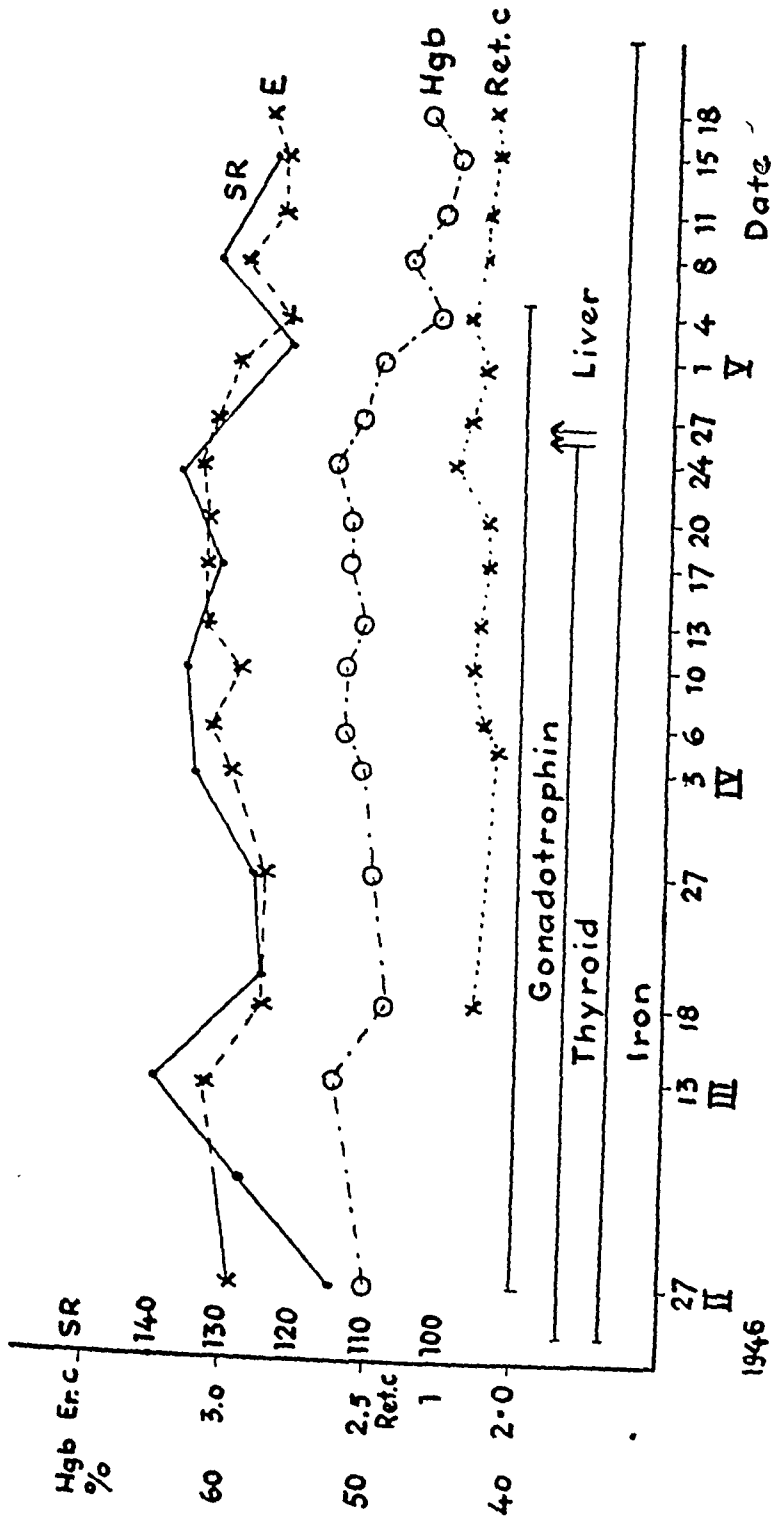


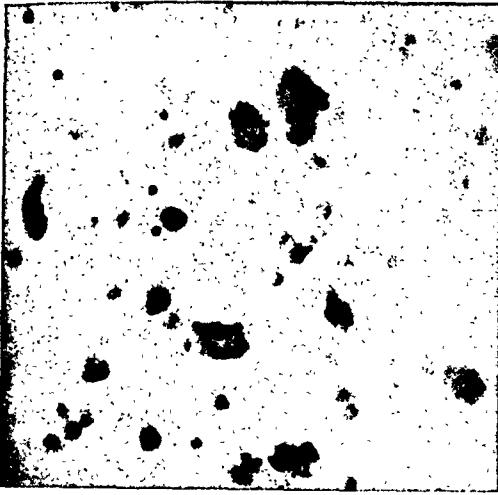
Fig. 2.

Blood sedimentation rate (SR), hemoglobin (Hgb), erythrocytes (E) and reticulocytes (Ret.c.) during gonadotrophin (21 injections), thyroid, liver and iron therapy.

on the erythropoiesis could be noted in spite of continued administration of iron in the usual doses together with or without dilute hydrochloric acid, liver therapy in the form of Heptomin forte Medica injections at three periods with 2 ampoules  $\times$  2, respectively 3 days after each other as well as combined with liver preparations per os, 3 injections Campolon at an interval of a week, Thyreoidin Medica in doses of 0.1—0.3 gm. 2—3 times a day for about 5 months as well as for periods, Ambinon Organon 15 ampoules for a month, testosterone propionate Neo-Hombreol Organon 18 ampoules of 25 mg. for 6 weeks together with Doca Organon 15 injections of 10 mg. and iron per os, a gonadotrophin preparation Antex Leo 21 ampoules for 5 weeks (the dose is unfortunately not indicated in the hospital records), Testoviron 10 mg.  $\times$  12 for a fortnight, etc. The hormone preparations were tried both alone and in combination with iron or liver. A, B, C and D-vitamins were also tried. The figures also revealed that the blood sedimentation rate and Hgb and Er-values respectively often ran parallel, though lower values for Hgb and Er with higher values for SR might well have been expected, since this is usual in chronic infections. It must be stated that the doses of liver and hormones may have been too small.

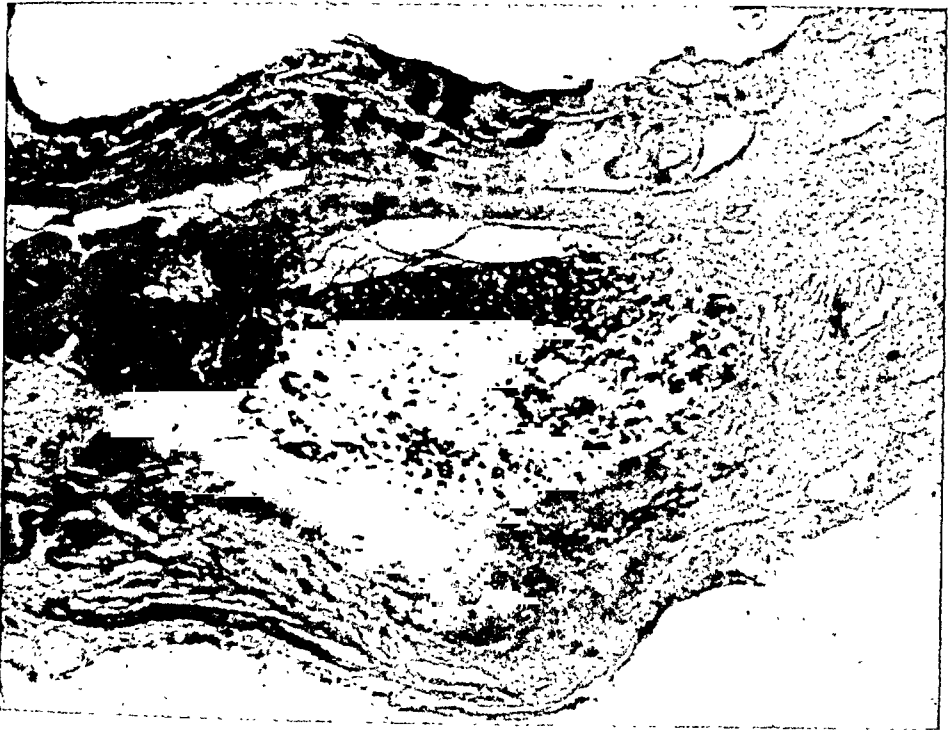
*Record of autopsy*, done by Professor I. Wallgren about 27 hours after death had occurred (brain and hypophysis were taken out and placed in 10 per cent formalin about 8 hours after death): Gyri of the brain were somewhat flattened. The formalin treated brain substance appeared macroscopically normal. In the ependyma of the IVth and IIIrd brain ventricles there was a slight fine granulation. The hypophysis looked like a transversal whitish string about 1 cm. long. Heart weighed 200 gm., otherwise normal. In the right lung there was much edema, the left one appeared normal. Liver weighed 1050 gm., otherwise normal. Kidneys weighed 200 gm., capsule somewhat adherent, surface smooth, structure plain. Stomach normal. Prostate and testes small, in aorta moderate sclerosis. Thyroid small, weighing 12 gm., firm with grey spotted surface on section. The bones of the skull and the right femur diaphysis were remarkably firm. In the sternum yellow bone marrow, in femur red.

Parts of hypothalamus and the region about aqueductus Sylvii. nucleus ruber and pedunculi cerebri embedded in paraffin wax were examined histologically. The sections were stained with hematoxylin-v. Gieson, toluidin blue and the marrow sheaths were stained according to a method suggested by Woelcke, 1942. Serial sections of the hypothalamus were made. In the hypothalamus no signs of



*Fig. 3.*

Ganglion cells in the region of nucleus paraventricularis (stained with toluidin blue). Magnification 240 X.

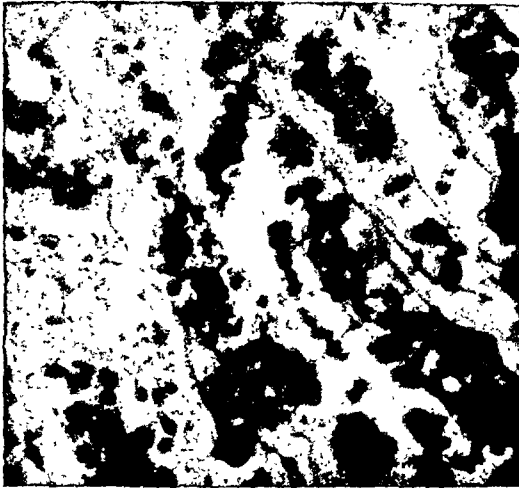


*Fig. 4.*

Cross section of hypophysis (stained with hematoxylin—v. Gieson).  
Magnification 31 X.



abnormal cell infiltration or of injury to the cell bodies were present. No ependyme granulations were noted. Substantia nigra was well preserved. Fig. 3 shows ganglion cells from hypothalamus from the proximity of the IIIrd brain ventricle. The cell body count in nucleus paraventricularis and nucleus supraopticus appeared to be somewhat reduced, but it was difficult to form an adequate opinion in this respect in a single case. — Serial sections of the pituitary body were made, all of which revealed considerable changes in this organ. Enormously increased connective tissue appeared especially in the periphery (Fig. 4). Most of the cells were small, dark, with a minimum of cytoplasm and without vacuoles, with round or oval nucleus. Only occasionally did larger cells of a lighter shade of eosinophilic or basophilic type occur. (Fig. 5). The anterior lobe



*Fig. 5.*

Cells of the hypophysis anterior lobe (stained with hematoxylin—v. Gieson). Magnification 240 X.

appeared to contain almost exclusively head cells. Some preparations revealed a tissue in the proximity of the anterior lobe consisting of fine red threads arranged in a net-like pattern with a few spool-shaped or star-shaped cells and brown refractive lumps. This was obviously part of an abnormal posterior lobe. Near this tissue single lymphocytic infiltrations appeared. — In the thyroid increased tissue and a good deal of lymphocytic infiltration were noted. Acini were poor in colloid, the cells cubical. — Liver and pancreas revealed normal histological structure. — The adrenals also revealed normal histological structure. In the prostate much smooth muscles and many cavities covered with cells and resembling sinus

structures in urethra. — Pathological-anatomical diagnosis: Fibrosis hypophysis et glandulae thyreoideae. Splanchnomicria. (The histological preparations were also examined by Professor I. Wallgren, and the preparations from the brain stem were also examined by Dr. K. v. Bagh, and I wish here to thank them for their courtesy). The cause of death was probably a general endocrine deficiency.

*Summary of the case history.* After a four months admission to hospital in 1928 in France and Germany, during which the patient had fever for nearly two months, there was hair loss, testis atrophy leading to impotence in 1942, lowered basal metabolic rate and other symptoms suggesting pituitary insufficiency. During this period a slowly progressive normochromic anemia with aplastic features was present. In consequence of a trauma in the left thigh in 1945, soreness and stiffness in the corresponding knee developed until complete ankylosis set in. The patient's condition became progressively worse. The anemia reached the lowest count 39/41 per cent for hemoglobin and 1.970 millions for erythrocytes. Attempts to affect the anemia produced no apparent results. The sexual function and progeria seemed to be affected by Doca + testosterone propionate. The patient died of general cachexia in 1947. Autopsy revealed splanchnomicria and an abnormally small hypophysis, transformed into connective tissue. Microscopic examination revealed marked fibrosis of the pituitary body and reduction of eosinophilic and basophilic cells in the anterior lobe. Increased connective tissue, though in a less degree, was also present in the thyroid. Hypothalamus appeared to be normal.

## DISCUSSION

Common symptoms of Simmonds' disease, all of which were present in this case, are asthenia, underweight, loss of hair, dry skin and, more rarely, skin pigmentation. Further signs are lowered basal metabolic rate and, probably as a consequence of this, increased sensitivity to cold. These symptoms were not affected by thyroid medication. In the present case thyrotrophic hormone was also administered in

the form of Ambinon Organon, without any apparent effect on the symptoms mentioned or on the blood picture. It should, however, be noted that the patient's general condition was then already very bad. It is interesting that desoxycorticosterone acetate in combination with testosterone propionate had a beneficial effect on the progeria as well as on the sexual function. One result of this was an erection, which had not occurred for several years. This is of interest when considering the relative efficacy of the doses in attempts to affect the patient's anemia. — The results of substitution therapy in Simmond's disease are on the whole negative, as indicated by cases confirmed at autopsy (*Escamilla & Lissner, 1942*), but good results with testosterone propionate, desoxycorticosterone acetate and thyroxin have recently been reported (*Luft & Sjögren, 1949*). — This patient also revealed obvious symptoms of disturbance of the carbohydrate metabolism of a type different from that associated with diabetes mellitus. This is not uncommon in Simmonds' disease (*Escamilla & Lissner, 1942*). — Symptoms of the nervous system such as apathy and incapacity for regular work as well as deficiency of facial and other movements have also frequently been observed in Simmonds' disease (*Sheehan, 1939*) and were also noted in this case. It might be assumed that the patient had suffered from an encephalitis localized to the brain stem in connection with his severe illness in 1928. The histological examination, especially of the hypothalamus, appeared to indicate that at least this region had escaped inflammatory processes. Considering the great change revealed in the hypophysis, it hardly seemed probable that the basal ganglia had been subject to changes caused by a chronic encephalitis, as the inflammatory process would then have »jumped over« the hypothalamus. The possibility of minor degenerative changes due to partial elimination of the pituitary body cannot, however, be excluded.

A predominant feature of the disorder during the final years was the *joint symptoms*. Are they manifestations of a chronic rheumatic disorder of the joints, independent of the

hypophysis, are they secondary symptoms of pituitary deficiency or are they possibly related to the trauma preceding the appearance of these symptoms? The fact that no real joint signs were observed during the years 1929—1945 in spite of the raised blood sedimentation rate and anemia is evidence against a chronic polyarthritis. Although the patient limped slightly and occasionally had diffuse pains in his legs, he had no fever and during his many admissions to hospital nothing would have justified the diagnosis of polyarthritis. It should, however, be kept in mind that a subnormal temperature is sometimes associated with Simmonds' disease (in 35 per cent of cases according to *Escamilla & Lisser*, 1942). Rather interesting is the fact that the joint signs were clinically observed and progressed rather quickly as a result of a trauma in 1945. *Jonsson & Berglund* (1949) have recently critically examined the relation between trauma and polyarthritis and among 2236 cases of polyarthritis they found only one in which a relation could be assumed. They believe, however, that in some cases trauma might be a contributory cause of polyarthritis. As in this case a possible chronic infection preceding the trauma must be taken into account, only secondary importance, if any, can be attributed to the trauma as a cause of the joint trouble. It is remarkable that with rather marked joint symptoms the patient had no fever during the whole of this post traumatic period. Even previous to the trauma a considerably raised blood sedimentation rate and anemia were present. If a relation between these signs and a chronic arthritis had existed, at least occasional fever or marked joint signs would probably have occurred during the earlier stages of the disease. This, however, was not the case. It should also be noted that the course of the blood sedimentation rate often ran parallel with or independent of the anemia (compare Figs. 1—2), whereas in cases of chronic polyarthritis an increased blood sedimentation rate is usually associated with worsening of anemia (*Nilsson*, 1948). It is thus difficult to interpret the occurrence of the ankylosing knee affection directly following

on a trauma in the left thigh unless the presence of an endocrine factor is assumed.

Patients with Simmond's disease often have rheumatic symptoms in their limbs and osteoarthritis has been proved *post mortem* (Sheehan, 1939), though by no means regularly. Escamilla & Lisser (1942) do not mention this symptom in a single instance of their large number of cases i.e. 101 confirmed at autopsy. Joint diseases of the type osteoarthritis deformans occur in acromegaly and Cushing's disease and they are also accompanying disturbances in conditions involving the brain stem and hypothyroidism (quoted by Lichtwitz, 1941, and Selye, 1948). Castration seems to predispose to joint diseases and it has been reported that testis preparations can prevent osteosclerosis brought about experimentally in animals by large doses of oestrogens (Jonsson, 1939). The anterior pituitary hormone may bring about bone and joint changes (Silberberg & Silberberg, 1940). Lowered secretion of oestrogenic hormones (Rasmussen, 1936) and ovarian failure have been noted in cases of chronic polyarthritis (Sjövall, 1944). The so called climacteric arthritis has probably an endocrine origin. Of interest is the recent research on the metabolism of joint cartilage. From the testes of animals an enzyme has been isolated which splits, among others the polysaccharide chondroitin sulphuric acid in joint cartilage (Meyer *et al.*, 1941). With such testishyaluronidase produced from fresh bull's testicles, it has been possible *in vitro* to affect cartilage from different joints taken from fresh autopsy cases so that histological pictures developed resembling those seen at beginning malacia in arthritis deformans (Hirsch, 1947). — Disturbances in the non protein nitrogen balance in connection with pituitary insufficiency (Lee & Ayres, 1936, and others) and reduction of the albumin-globulin quotient after hypophysectomy in animals have been described, (Podhradzsky, 1940), but are not mentioned in human by a number of investigators. A markedly raised blood sedimentation rate without joint symptoms has been reported in a case of atrophy of the hypophysis (Jersild, 1943).

The experimental investigations up to date appear to suggest a relation between joint changes and endocrine disturbances. In the present case many features differed from the symptomatic picture of rheumatic polyarthritis. The severe endocrine hypofunction revealed in the patient would appear to indicate that this failure was a contributory cause of the

joint disease activated by the trauma. It is, however, hardly possible to discover the exact mechanism with data of a single case.

In this case the *anemia* also deserves detailed discussion. An account of earlier investigations on this subject is first given.

From the relevant literature, *Sheehan* (1939) has compiled 31 cases of Simmonds' disease, caused by necrosis of the hypophysis associated with partus, in which attention has been paid to the blood picture. Of these, 19 revealed anemia and 4 polycythemia while the rest had a normal red blood picture. A table given by *Sheehan* further indicates that in 9 cases genital atrophy without anemia was present, while 12 cases with genital hypoplasia revealed anemia and 1 case with normal genitalia showed a normal blood picture. All cases of polycythemia had genital atrophy. This survey thus reveals no clear correlation between blood picture and genital function. *Sheehan* further states that in the material described by him the erythrocyte values during the first five years of the disorder remained about 5—6 millions and the colour index varied between 0.6 and 0.8. During the following five years the corresponding values were 3—4 millions and 0.7—0.9, while in cases of longer duration the number of erythrocytes could be reduced to 2—3 millions and the colour index to 0.95—1.25. He indicates that the hyperchromic anemias are more frequent in cases with signs of hypothyroidism. The leucocyte values were normal. Often a relative lymphocytosis and eosinophilia were noted. Among 6 cases of pituitary insufficiency (*Snapper et al.*, 1937), which included 4 males with hypogonadism as the predominant sign, 5 had achylia and 4 certainly had hypothyreosis, degeneration of the spinal cord of the type funicular myelosis was present in 2 cases, anemia of pernicious type in 2 and hypochromic anemia in 1. Three of the cases were cured by liver therapy, one by the administration of thyrotrophic and gonadotrophic hormone and the hypochromic anemia with the same hormones in addition to iron. A case of hypogonadism and hypochromic anemia has also been described in which preparations of testis and gonadotrophic hormone in combination with iron had a beneficial therapeutic effect while synthetic preparations had none (*Gonnermann*, 1938), 1 case of a hyperchromic type with pituitary deficiency reacted to liver (*Witts*, 1942), in 1 case of hyperchromic anemia and 1 of a hypochromic type, testosterone propionate in combination with liver, or iron, had a beneficial effect (*Watkinson et al.*, 1947). A number of other similar cases have been described (*Foster & Mc Carter*, 1941, *Williams &*

*Whittenberger*, 1942). In castrates a slight anemia has been observed (*Mc. Cullagh & Jones*, 1942). In a survey of 101 cases of Simmonds' disease confirmed at autopsy *Escamilla & Lissner* (1942) give the average values 65 per cent (min. 40 per cent) for hemoglobin and 3.710 (min. 2 millions) for erythrocytes. The type of anemia is not mentioned in the paper. Of special interest from the point of view of the present case is an observation on a patient with aplastic anemia and with a cyst destroying the hypophysis (*Bloom & Bryson*, 1948). As is well known, polycythemia has been reported in cases of pituitary disturbance such as Cushing's disease and acromegaly. This question has, however, never been thoroughly analyzed. It is possible that dehydration could be demonstrated in these instances. (*Snapper et al.*, 1937). — In animal experiments some facts have recently been noted which appear to indicate that androgenic hormones stimulate the erythropoiesis (*Steinglass et al.*, 1941; *Finkelstein et al.*, 1941; *Arendsen de Wolff-Exalto*, 1947). — In connection with hypothyreosis, hypochromic and, though more rarely, hyperchromic anemia have been described. The beneficial effect of thyreoidin on anemias of this type is considered characteristic (*H. Zondek*, 1922; *Unverricht*, 1923; *von Boros & Czoniczer*, 1935; *Holboll*, 1931, and others). Hypothyroidism and anemia in Simmonds' disease are on the other hand not as a rule affected by thyroid preparations (*Escamilla & Lissner*, 1942).

Animal experiments have shown that hypophysectomy in rats causes a reticulocytopenia, which regenerates after about a month (*Overbeek & Querido*, 1939; *Meyer et al.*, 1940; *Ruitinga et al.*, 1940), as well as anemia (*Wilson*, 1937; *Vollmer et al.*, 1939; *Meyer et al.*, 1940; *Crafts*, 1946). Hypophysectomy in the rabbit led to the same result (*Houssay et al.*, 1931). The reticulocytopenia has been attributed to decreased erythrocyte destruction (*Overbeek & Querido*, 1939), but this opinion has been subject to criticism (*Meyer*, 1940). *Flaks et al.* (1938) report that they have found a factor which stimulates the formation of erythrocytes and reticulocytes in an extract from the anterior lobe of the pituitary gland, free from growth, gonadotrophic or thyrotrophic hormone. This extract exerts its action only with oral administration. *Meyer et al.* (1940) could not confirm *Flaks'* findings, but they were of the opinion that the preparation used by them possibly lacked *Flaks' factor*. They believe, that the changes in the red blood picture caused by hypophysectomy cannot be attributed to the lack of some specific hormone in the pituitary gland regulating the hematopoiesis, but are due to general metabolic changes associated with hypophysectomy. *Crafts* (1946) showed that the anemia was microcytic and hypochromic and he was able to prevent it with injections of thyroxin, iron and copper.

The change in the leucocyte picture seems to be slight in the above mentioned experiments involving hypophysectomy. In a recent survey, *Doughaday et al.* (1948) are of the opinion that the erythropoiesis is not subject to primary hormonal control.

The facts observed appear to indicate that hypogonadism with or without other signs of pituitary deficiency may in some cases cause an anemia which can be successfully treated with gonadotrophic hormone. On the other hand the type of anemia described in connection with Simmonds' disease appears as a rule to be unaffected by hormone therapy. A case of aplastic anemia associated with injury to the hypophysis has recently been described. A number of animal experiments indicates the possibility that the anterior lobe of the pituitary contains specific hormones regulating erythropoiesis, but the evidence of these experiments has been questioned. In any case it cannot be denied that the anterior lobe might contain specific erythropoiesis regulating substances. The facts observed during the progress of the anemia in the present case, particularly the absence of association between the blood sedimentation rate and the joint signs, the aplastic character of the anemia and the marked signs of pituitary failure, would appear to indicate the existence of a relation between the blood disease and the endocrine disturbance. The presence of increased connective tissue in the pituitary body and to some extent also in the thyroid seemed to suggest a previous inflammatory process, but no signs of actual inflammation were present at the time of post mortem examination. Consequently, a process of this kind could not have been a decisive cause of the raised blood sedimentation rate and the anemia. This view is also supported by the presence of a normal serum iron. — A cerebral factor must also be taken into account. A number of clinical experiments and animal experiments (literature given by *Hortling*, 1948) indicate the possibility that parts of the hypothalamus, perhaps in the first place in the nucleus paraventricularis, and some adjacent areas (*Hess*, 1947) play a part in the regulation of the erythropoiesis. The histological structure of hypothalamus ap-



peared, however, to be normal in this case. Nevertheless it is possible that the number of ganglion cells in the nuclei of the hypothalamus was reduced, as has been observed when the pituitary stem has been surgically removed (*Rasmussen & Gardner, 1940*).

The posterior lobe of the pituitary body in this case showed pathological changes although no clinical signs had been present. Signs of diabetes insipidus were not observed. It should, however, be kept in mind that only 15 per cent of the ganglion cells in nucleus supraopticus are required to prevent diabetes insipidus (cf. *Rasmussen & Gardner, 1940*). Injection of the posterior lobe hormone pituitrin in the rabbit and guinea-pig is reported to cause anemia of a hemolytic type in some cases (*Dodds et al., 1936*).

Interesting in this case is also the great reduction of the basophilic and eosinophilic cell counts in the anterior lobe of the pituitary body, while cells of a type resembling head cells were predominant. Similar observations in cases of pituitary fibrosis have previously been made (*Langdon-Brown, 1936; Doane et al., 1940; Jersild, 1943, and Øllegaard, 1945*). It should be mentioned that increased connective tissue was also noted in the thyroid, suggesting the possibility of a systemic disorder of the endocrine organs. This has previously been described in single cases (quoted by *Escamilla & Lisser, Jersild and Øllegaard*).

### SUMMARY

A case of pituitary insufficiency is described, which besides having the classical signs of Simmonds' disease, revealed an ankylosing joint affection of the left knee, developing after a trauma in the left thigh, chronically raised blood sedimentation rate already present before the appearance of the joint disorder and a normochromic anemia with aplastic features. At autopsy, splachnomicry and an abnormal hypophysis were noted. Microscopic examination revealed considerably increased connective tissue in the hypophysis and moderately increased connective tissue in the thyroid. In the anterior lobe

of the hypophysis cells resembling the head cells were almost exclusively observed. The relation between the joint condition and anemia on the one hand, and the pituitary failure on the other, is discussed; it is considered that such a relation may exist.

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## HYPERPARATHYROIDISM COINCIDENCE OF PARATHYROID ADENOMA, GOITER AND EXOPHTHALMOS. REPORT OF A CASE

BY

SVERRE AARSETH and EINAR BJØRGO

### INTRODUCTION

It is now about a quarter of a century since *Mandl* removed a parathyroid adenoma in a patient with osteitis fibrosa generalisata, and thus obtained the final proof of the causal relation between these conditions. Since then, a great many cases with this condition, to which *Barr & Bulger* (1930) applied the term hyperparathyroidism, have been reported. *Norris*, in 1947, presented a survey of 322 cases of parathyroid adenoma collected from the world literature, and since then several additional cases have been published. According to *Hagtvet*, about 30 cases of hyperparathyroidism have been observed in Norway. The various clinical manifestations of the disease are now generally recognized, and the diagnosis, which is based primarily on comparatively simple calcium and phosphorus analyses in blood and urine, rarely involves serious problems. On the other hand, the etiology of primary hyperparathyroidism is far more complex and is not fully known.

The following case of hyperparathyroidism is therefore reported for the purpose of contributing to the discussion of the

etiology of the disease. Moreover, in this patient a severe, protracted tetany which gave rise to spontaneous fracture of the neck of both femurs was observed after the operation.

### CASE REPORT

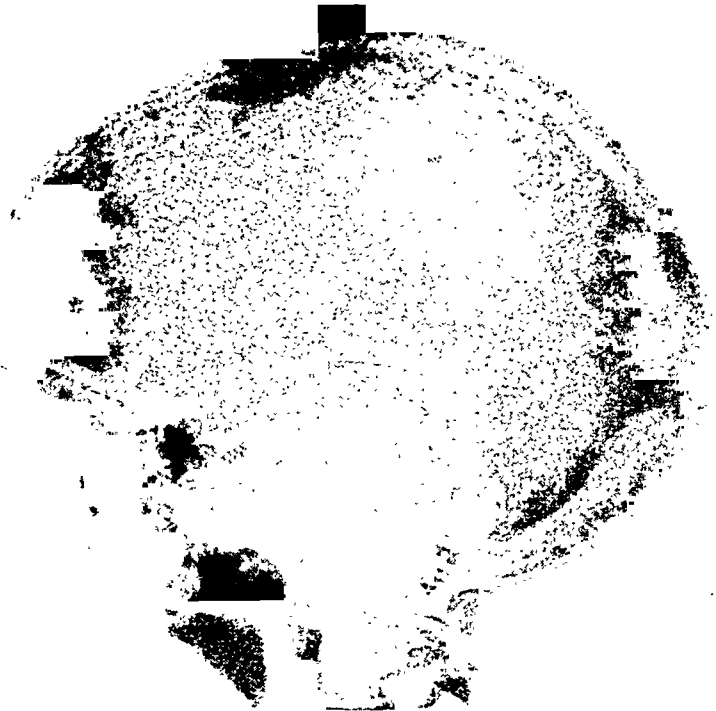
*Past History.* A. F., a 29 year old unmarried woman, at the age of 20 suffered from left-sided pleurisy. About the same time a severe alveolar pyorrhea developed, for which reason all the teeth in the upper jaw were extracted. In 1946, i.e., at the age of 28, loosening of the teeth in the lower jaw occurred, and these teeth were also extracted. Towards the summer of the same year she constantly felt tired and indisposed. However, she continued her work as manageress of a small hotel. In December (1946) she felt worse, and therefore consulted a physician who diagnosed a moderate anemia (Hb 70 per cent). Iron and liver medication was instituted, and a transient improvement of the anemia was observed. Her general condition, however, did not improve but became steadily worse. An increasing loss of strength developed in the arms and legs, and the general weakness also increased. She lost her appetite completely and had an excessive thirst. She also noticed that since the spring of 1946 she had become more round-shouldered, and she had aching pains in both thighs, legs, knees and in the left wrist.

*Physical Examination.* On April 25, 1947, she was admitted to the Medical Department for observation. She was of an average weight, slightly round-shouldered, and slightly built. The nails were remarkably white. Her complexion was pale. A slight exophthalmos was present. The thyroid gland was moderately enlarged without palpable nodules. Otherwise, routine clinical examination revealed nothing abnormal, as did the neurologic examination.

*Laboratory Data.* Meinicke's test was negative. The urine was turbid, and contained albumin (0.025 per cent). The specific weight was 1024. Microscopic examination of the urine revealed numerous pus cells, and cultivation gave growth of *E. coli*. Sulkovitch's test was positive. The hemoglobin content was 80 per cent, the white cell count 5,200, and the red cell count 3.6 millions. A differential count was normal. The icterus index (Meulengracht) was 4 units, and the sedimentation rate 35 mm. The non-protein nitrogen was 25 mg/100 ml. The Takata-Ara test was negative. The serum proteins (Bing) were 5.5 per cent, the calcium content in serum, 14.8 mg/100 ml., the phosphorus content, 2.3 mg/100 ml., and the content of alkaline phosphatase, 42.7 Bodansky units. A sugar tolerance test showed a normal blood sugar curve. The basal metabolism varied between 109 and 103 per cent. An ECG showed a sinus rhythm of

100, and a PQ interval of 0.23 seconds, a QT interval of 0.32 seconds (normal values 0.29—0.36), lowered T waves and slight depression of the S-T interval in the first and second leads.

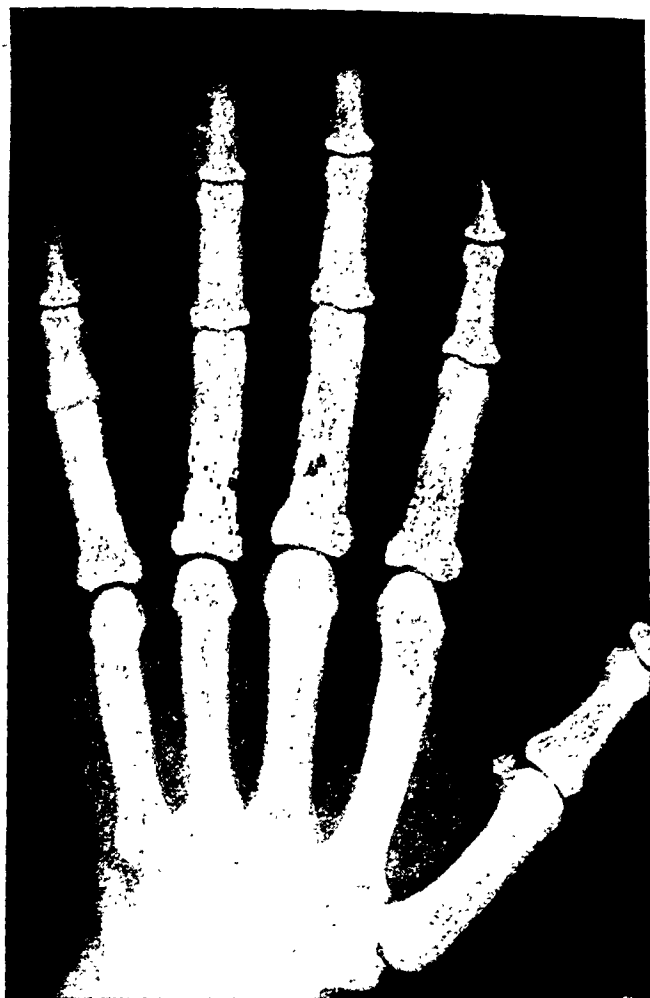
*Röntgenographic examination* of the skeleton (Dr. K. Oppegård) showed extensive changes in most of the bones with evidence of general decalcification. The bone structure varied, giving the bony tissue a fuzzy, worm-eaten, or granular appearance, with almost



*Fig. 4.*

Roentgenogram of the skull before the operation.

complete decalcification in some areas. Some of these decalcified areas were comparatively well defined and appeared as cyst-like, punched-out areas. The changes were seen to involve the entire bone or portions of a bone with diffuse transition into normal bone tissue. The most extensive changes were seen in the skull, the facial bones, the forearms and hands, but there was considerable involvement also of the pelvis and the shoulder girdle. The spinal column showed osteoporosis, but no evidence of destruction of vertebrae. In the regions of both kidneys numerous pin-point sized calcium deposits were seen. The deposits were arranged in groups corresponding to



*Fig. 2.*

Roentgenogram of the right hand before the operation.

the calyces and renal papillae. Intravenous pyelography showed adequate excretion of contrast medium on both sides. No evidence of dilatation of the urinary passages was observed. (Figs. 1—4).

The condition was diagnosed as hyperparathyroidism and the patient was transferred to the Surgical Department on May 16, 1947, where extirpation of a parathyroid tumour on the left side and subtotal thyroidectomy was performed on June 11, 1947 (Dr. Kr. Stray) under morphine-ether-nitrous oxide anesthesia. A bilobar goiter was present. Further, a grape-sized, encapsulated, well de-

finer, ellipsoid tumour was found at the lower pole of the left lobe of the thyroid. The tumour was of a darker red colour than that of the enlarged thyroid. It was removed whole without severing the capsule. Further, a piece shaped like an orange section was excised from both thyroid lobes.



*Fig. 3.*

Roentgenogram of the right shoulder before the operation.

The excised tumour, including the capsule, weighed 4 gm. and measured 2.8 by 1.8 by 1.5 cm. The capsule was very thin and was composed of fibrous tissue. The surface was smooth without nodules. The cut surface had a homogeneous light brown, somewhat fatty, glistening appearance. In some areas, centrally as well as peripherally, small hemorrhages were seen. The consistency was firm-elastic. The pieces of thyroid tissue removed weighed 10 and 12 gm., respectively. They had a slightly bulky, capsule-covered outer surface which showed no evidence of adenoma. The cut surface was



brownish red, partly transparent and showed slight fibrosis, particularly in the central areas.

*The histological examination* (Prosector Dr. Erik Waaler) showed a rather uniform structure of the encapsulated tumour, which consisted mainly of cuboid to polyhedral epithelial cells arranged in columns which in some areas showed a tendency to alveolar group-



*Fig. 4.*

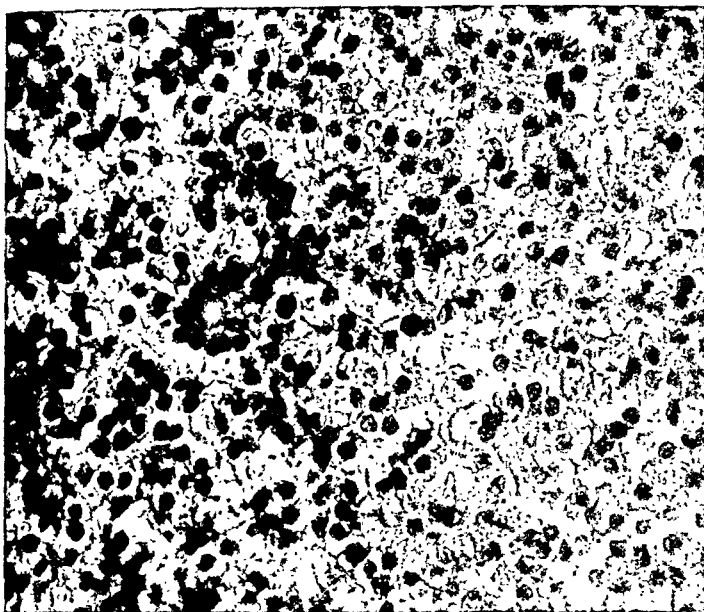
Nephrocalcinosis. Extensive involvements of both kidneys.

ing. The cells had a lightly staining cytoplasm (typical waterclear cells). The nuclei were round, with medium chromatin content, and on the whole evenly stained. Evidence of malignancy was not revealed, and the patho-anatomic diagnosis was parathyroid adenoma (Fig. 5).

Microscopic examination of the two pieces of thyroid tissue showed rather large glandular alveoli lined by a low cuboid epithelium and filled with colloid substance. Toxic changes were not demonstrated.

*Postoperative Course.* The primary postoperative course was uneventful and the wound healed by primary union. On the twelfth postoperative day, however, tetany with convulsions developed. The

next day the patient complained of stiffness in both lower extremities. She was then transferred to the Medical Department. On the fifteenth postoperative day the blood calcium was only 5.2 mg./100 ml. despite intravenous administration of large doses of calcium. A. T. 10 was administered as long as this drug could be obtained. Later she received doses of Afi D<sub>2</sub> Forte (250,000 units per day). However, increased reflex activity and the peroneal signs persisted,



*Fig. 5.*

Section from the parathyroid tumour. Preponderance of water clear cells.

and Chvostek's sign was present on a single occasion. The blood serum calcium content showed a steadily decreasing trend. The lowest value, viz. 3.6 mg./100 ml. was observed on June 7, 1947, i. e., 27 days after the operation. On Aug. 23, 1947, i. e., 73 days after the operation, the calcium content was still very low, viz., 4.7 mg./100 ml., while the phosphorus content was normal, and the alkaline phosphatase content had fallen to 9.6 Bodansky units. During the subsequent period the blood calcium content rose rapidly and at times reached hypernormal values. The calcium, phosphorus and phosphatase values are shown in the diagram (Fig. 6).

The patient had a persistent anemia which did not respond to iron medication, and blood transfusions were therefore given. The ECG showed a prolonged QT interval.

Roentgenographic examination on Sept. 9, 1947, showed a definite increase of the calcium content of the bones. The examination also revealed fracture of the neck of both femurs. These fractures had not been clinically diagnosed and it cannot be said at which stage of the postoperative period they occurred. No doubt, however, they occurred during the attack of convulsions (see diagram, Fig. 6).

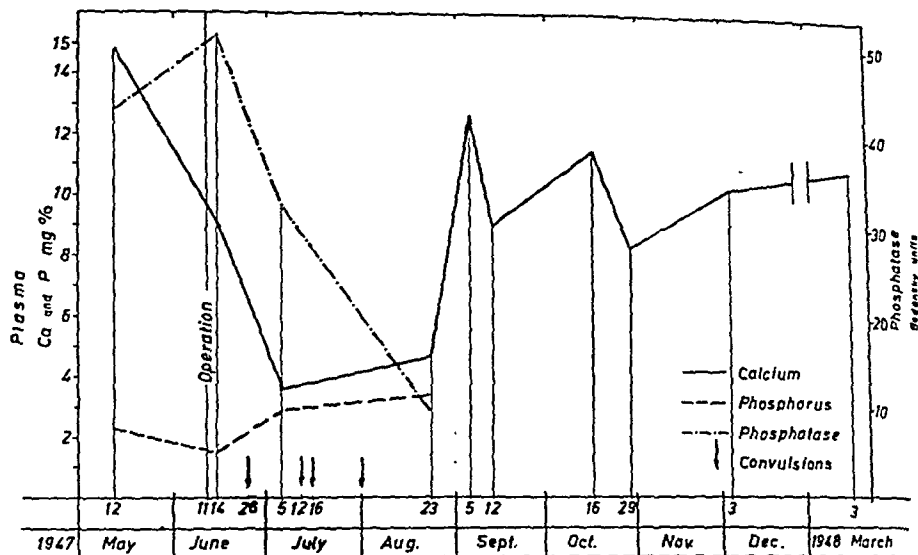


Fig. 6.

Calcium, phosphorus, and phosphatase values in the blood before and after the operation. Attacks of convulsions indicated by arrows.

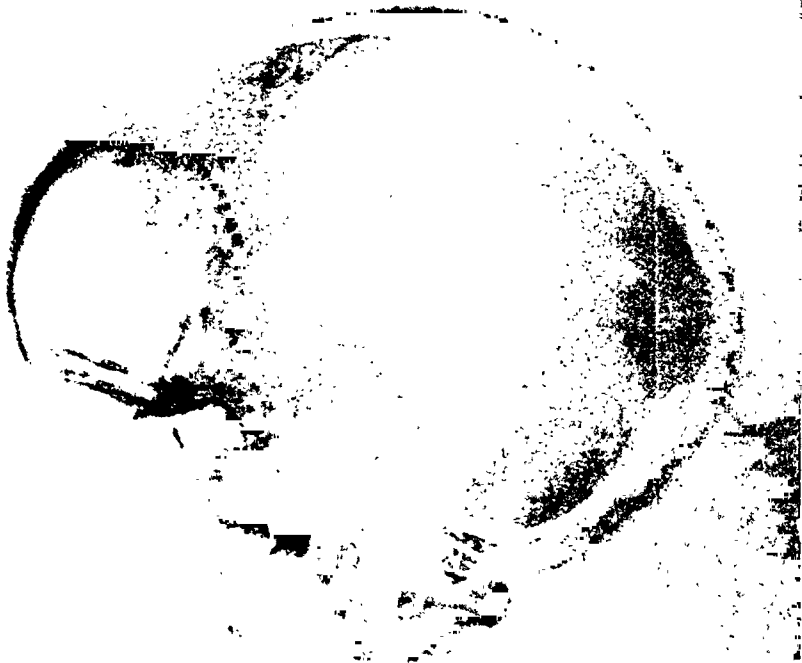
She was re-transferred to the Surgical Department on Sept. 13, 1947, and a bilateral wire traction through both tuberositates tibiae was applied. After two months there was still a definite line of separation. Massage in bed was instituted and was continued for several months. About five months after the operation she was able to walk on crutches. Calcium preparations were given all the time, and an evenly increasing calcification of the skeleton was observed. The patient was discharged on Dec. 12, 1947, feeling well.

On re-examination Jan. 16, 1948, she was able to walk without sticks. Roentgenograms of the femurs showed satisfactory callus formation on both sides.

Feb. 10, 1948: The patient has gained considerable weight. The gait is improved, though waddling. She feels well. March 30, 1948: The gait is further improved, though still slightly waddling. The patient feels perfectly well. Blood: Hb 78 per cent; RBC 3.74 millions;

WBC 5,000; sedimentation rate 11 mm.; non-protein nitrogen 30 mg./100 ml.; serum calcium 11 mg./100 ml. *Urine*: Slight albuminuria, but no blood or pus. ECG normal.

Roentgenographic examination on *March 30, 1948*, showed additional calcification giving the bones a more normal appearance,



*Fig. 7.*

Roentgenogram of the skull 292 days after the operation. Compare with Fig. 1.

particularly the bones of the fore-arms and hands. The fractures were completely healed, while the calcium deposits in both renal regions persisted (Figs. 7—9).

### COMMENTS

Under normal conditions the ductless glands form an inter-related system. Most of the glands are influenced by the organotrophic pituitary hormones, several of which have been isolated, e. g., the adrenocorticotrophic hormone, gonadotrophic hormones, and thyrotrophic hormone. The latter is probably of essential importance in the pathogenesis of hyperthyroidism. Similarly, the exophthalmos in Basedow's disease



*Fig. 8.*

Roentgenogram of the right hand 292 days after the operation.  
Compare with Fig. 2.

is assumed to be due to the action of an ophthalmothyrotrophic hormone produced in the hypophysis (*Wijnblad*, 1948.)

Similarly it has been suggested that primary hyperparathyroidism might be due to the action of a parathyrotrophic pituitary hormone. This view is supported by several clinical, pathological and experimental observations.

A simultaneous occurrence of hyperparathyroidism and hyperthyroidism strongly suggests a common pituitary genesis. *Gutman* (1937) thus claims to have observed a »well developed hyperthyroidism« in some cases of hyperparathyroidism. *Gilligan* (1934), in several patients with exophthalmic goiter,



*Fig. 9.*

Roentgenogram of the right shoulder 292 days after the operation.  
Compare with Fig. 3.

has found an increased content of parathyroid hormone in the blood. *Ponteva* (1939), in a 14 year old girl with exophthalmic goiter, found signs of a hyperparathyroidism, and *Bergstrand* (1931), in a case of goiter, found enlarged parathyroids. One patient observed by *Hellström* (1935) suffered from thyrotoxicosis, exophthalmos, and osteitis fibrosa generalisata. Autopsy in this patient revealed considerable changes in the hypophysis. *Means* (1948) reports that in 4 patients in whom

the parathyroids were incidentally removed in connection with a subtotal thyroidectomy, small parathyroid adenomas were found in 2 of the patients who had not presented clinical evidence of hyperparathyroidism. Autopsy in a 63 year old woman who suffered from osteitis fibrosa generalisata revealed a diffuse hyperplasia of the basophile elements of the anterior lobe of the pituitary gland, a large parathyroid adenoma, and a macro-microcellular adenoma of the thyroid gland (*Franck & Hjerrild, 1937*). *Eitinger* (1942) assumes that the so frequently occurring psychic disorders in hyperparathyroidism may be related to the presence of a pituitary tumour, and *Mellgren* (1945) and *Wilton* (1946, 1947) have found characteristic histological changes in the anterior lobe of the pituitary gland in 5 cases of hyperparathyroidism. *Wilton* believes that some cases of osteitis fibrosa generalisata are due to a hyperfunction of the hypophysis. *Anselmino, Hoffmann & Herold* (1933, 1934) in experiments were able to produce hypertrophy of the parathyroids associated with skeletal changes resembling those seen in osteitis fibrosa generalisata by the administration of anterior pituitary hormone.

*Törnblom* (1949) claims that the hypophysis affects the parathyroids via the blood phosphorus, and his experimental investigations do not indicate the presence of a parathyrotrophic hormone produced by the hypophysis.

In our patient colloid goiter, exophthalmos, and parathyroid adenoma associated with extensive skeletal changes were present at the same time. It seems justifiable to consider these conditions as related to a common hypophyseal origin. In our opinion the case supports the theory that the cause of hyperparathyroidism is to be found in the hypophysis.

The highly protracted course of the postoperative tetany which was observed in our patient must be considered in relation to the profound skeletal changes and the high phosphatase values prior to the operation. After the operation the decalcified bones will absorb calcium from the plasma to such an extent that hypocalcemia occurs. *Cope* (1941) in this connection speaks of »the hungry bones«. It must also be assumed

that a prolonged hyperactivity of one parathyroid leads to a suppression of the function of the other glands, so that it will take some time until normal functional conditions are established. Moreover, even if there are as a rule four parathyroids, this is not always the case. According to *Morgan* (1936) four parathyroids are found in 70 per cent, three glands in 24 per cent, two glands in 5 per cent, and a single gland in 1 per cent of all individuals. If an individual has merely a single gland which is the site of tumour formation or hyperplasia, and this gland is removed, permanent tetany will obviously occur. Further, aberrant parathyroid tissue may be incidentally removed when a partial thyroidectomy is performed.

*Conway* (1949) has recently published a case in which postoperative tetany occurred, and where blood transfusions gave rise to severe convulsions. The reason for this is assumed to be that citrated blood when used for transfusion fixes the free calcium ions in the blood with the result that a further decrease of the blood calcium content occurs («citrate tetany»). Our patient received two blood transfusions during the postoperative period when the blood calcium was very low, but convulsions were not observed. However, the possibility mentioned above should be borne in mind.

### SUMMARY

A case of hyperparathyroidism in a 29 year old woman is reported. Extensive skeletal changes and nephrocalcinosis were present. Furthermore, the patient suffered from exophthalmos and goiter. Operation revealed a parathyroid adenoma of the water-clear cell type, and colloid goiter. Postoperatively, a severe, protracted tetany and excessively low blood calcium values were observed. During an attack of convulsions, fracture of the neck of both femurs occurred. Considerable improvement was observed about ten months after the operation. The nephrocalcinosis, however, persisted. The simultaneous occurrence of hyperparathyroidism, goiter, and exophthalmos seems to support the possibility of a common hypophyseal origin.



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## A STUDY OF THE NERVES OF THE THYROID GLAND AND THEIR RELATIONSHIP TO GLANDULAR FUNCTION

BY

HJALMAR HOLMGREN and BENGT NAUMANN

### INTRODUCTION

That the thyroid gland has a rich nerve supply had already been demonstrated by the middle of the nineteenth century. As the nerves followed closely the circulatory system they were given a purely vasomotor function. *Poincaré* suggested in 1875 that the nerves had a direct effect on the secretory epithelium. Around 1890 interest was aroused and attempts were made to reach a final conclusion concerning this problem which has since been studied by a number of investigators and with various results. A short review of the morphological and physiological experiments which have been made will be given here.

*Bräeucker* (1923), who studied the innervation of the thyroid gland macroscopically, found that the thyroid nerve plexus is built up of vagus branches as well as branches from all three cervical sympathetic ganglia. He also found connections with the pharyngeal plexus (where the glossopharyngeal nerve enters). *Nonidez* (1931 b) considered that the innervation of the thyroid gland is composed principally of a branch from the superior laryngeal nerve, a branch from the cranial portion of the cervical sympathetic ganglion and perhaps bran-

ches from the other two cervical ganglia. *Rossi & Lanti* (1934/35) found that the nerve-fibres enter the parenchymatous tissue partly with the blood vessels and partly through the capsule.

Only a few microscopic experiments concerning thyroid innervation will be described.

*Andersson* (1894), *Rossi & Lanti* (1934/35) and others found that a perifollicular network divided itself from the nerves around the blood vessels and in some cases terminated with end plates near the thyroid cells, but do not consider that they have found positive evidence of a secretory innervation. *Nonidez* (1935), however, found no perifollicular nerve-endings and considers, in agreement with many other investigators e. g. *Eger & Titze* (1943) that positive secretory nerve-fibres do not exist.

After extirpation of the sympathetic system *Tronconi* (1937) and others claim to have found signs of degeneration or hypoactivity in the gland, a statement which *Vogt* (1931), *Brock, Doty, Krasno & Ivy* (1940) and others deny.

*Haney* (1932) demonstrated an increase in the metabolic rate after stimulation of the cervical sympathetic system. *Cannon, Binger & Fitz* (1914) and later *Friedgood & Cannon* (1940) were able to produce hyperthyroid symptoms in cats by faradic stimulation of the cervical sympathetic system and by phrenicosympathetic anastomosis. These results could not be confirmed by *Burget* (1917), *Marine, Rogoff & Stewart* (1918) and others.

*Uotila* (1939) believes that the cervical sympathetic system affects the thyroid gland only via the hypophysis. He also observed, after experimental severing of the stalk and the cervical sympathetic that the hypophysis possesses a secretory basal rhythm, which is, however, affected by impulses via the hypophyseal stalk and the cervical sympathetic as well as by humoral factors.

*Helin & Zilliacus* (1941) demonstrated a change in potential in the thyroid gland (electrothyreogram), a decrease in thyroid iodine and an increase in blood iodine as early as a few hours after faradic stimulation of the cervical sympathetic nerve in

a decerebrated cat. They interpreted their results as a sign, that secretion is at any rate partly dependent on the sympathetic system. Even after such a short period, however, thyrotrophic action cannot be ruled out (*Borell* 1945).

Thus it may be concluded that no evidence of secretory innervation of the thyroid cells has been found by either histological or physiological examination.

Some investigators consider that thyrotrophin is secreted by the hypophysis into the blood (*Westman & Jacobsohn*, 1938) while others (*Borell*, 1945) have drawn attention to the fact that the hormone may be carried up through the stalk of the hypophysis to higher regions such as the tuber cinereum.

*Our problems were as follows:*

1) What is the origin of the thyroid nerves and how do they stain with methylene-blue? Are the nerves only blood-vessel nerves?

2) Further investigation as to the effect of denervation on the histological picture and activity of the gland.

3) In view of 1) and 2) would any trade preparations affect the thyroid gland.

## MATERIAL AND TECHNIQUE

### *Staining methods.*

*Andersson* as early as 1894 tried to stain nerve fibres in the gland with methylene-blue, according to *Ehrlich's* method. Despite the poor results reported by *Andersson* this method has been chosen as it seems to give a picture that is easier to interpret than other methods and as it has been much developed since 1890 (*Schabadasch*, 1935, *Hillarp*, 1946). These authors state that the pH and the composition of the staining solution must be modified according to the organ to be stained, which is borne out by our staining experiments. After a series of experiments performed in order to obtain maximum staining of the nerves in a normal thyroid gland, the following composition of the injection material was found to be the most suitable: methylene-blue 0.10—0.010 gm., NaCl 7.25 gm., glu-

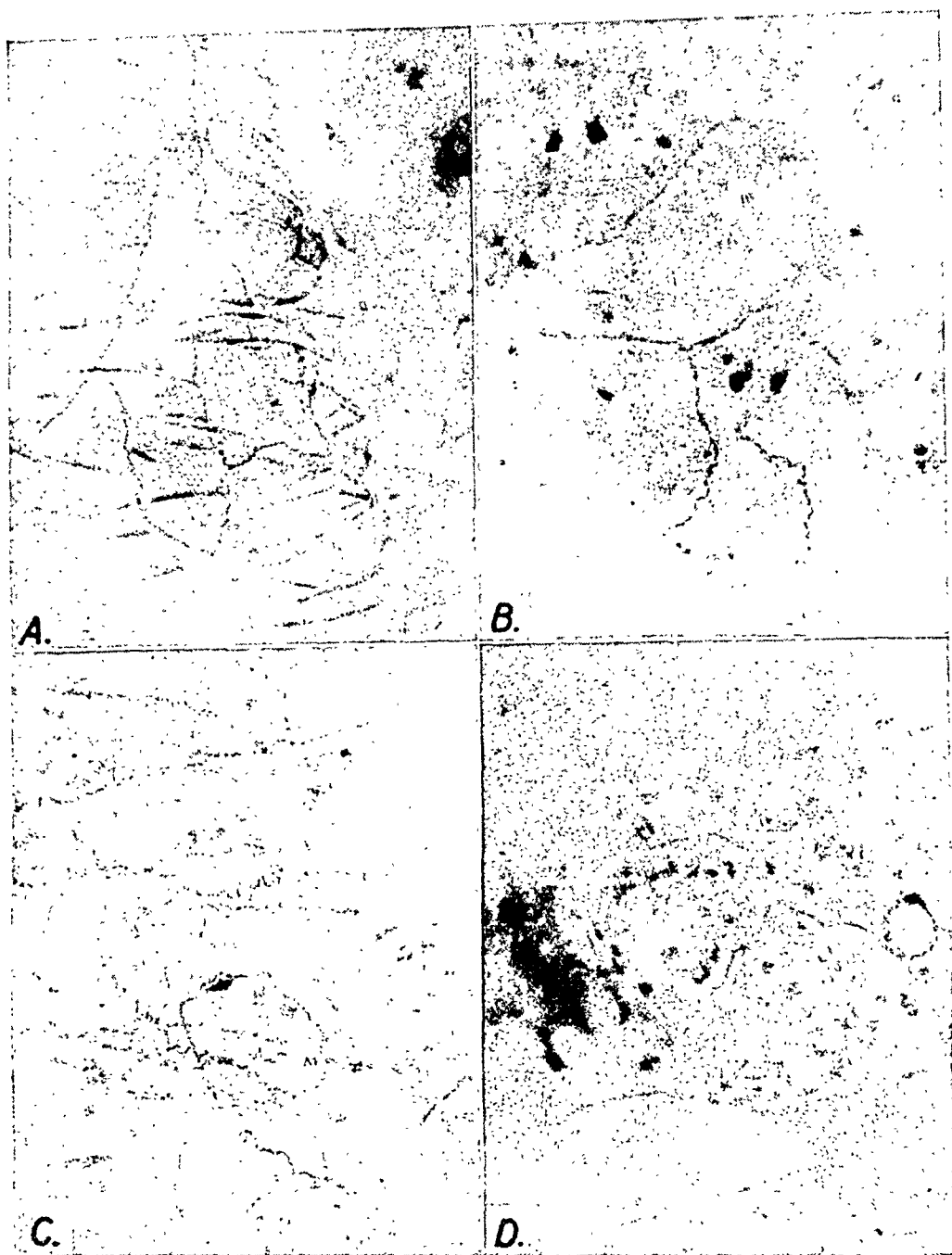
cose 2.0 gm., sodium acetate 0.3 gm., resorcinol 0.3 gm.,  $\text{MgCl}_2$  1.25 gm., aqua redest. 1000 gm.

A buffer solution (prim. and sec. phosphate) was added to give a pH of 6.10—6.30. The solution was heated to 50°C before adding the methylene-blue. The solution used was never more than 3 days old.

250—450 ml. of the staining solution (at 37° C) was injected into the thoracic aorta of the guinea pig (in an animal weighing 300 gm. e. g. 400 ml. solution, with 0.010 gm. of methylene-blue per liter) under a pressure of about 100—150 mm. Hg. This pressure caused a noticeable edema in the connective tissue. The injection time was 5—8 min. None of the large blood vessels in the area were ligatured. The right ventricle of the heart was opened to allow outflow of the solution.

After the injection, the thyroid gland, which was usually at that stage of a pale greenblue colour was taken out and placed in a solution of the same composition as the injection fluid, except that it did not contain methylene-blue. After five minutes the gland was transferred, still whole, to the fixing solution. This was a 2.11 per cent water solution of  $\text{NH}_4\text{J}$  plus ammonium picrate ad satur. The best fixation time was 15—20 hours, after which time the glands were placed in a mounting solution for a period of 24 hours. This solution was composed of chemically pure glycerin plus ammonium picrate ad satur. As methylene-blue stain cannot withstand the usual paraffin embedding and slicing and as gelatin embedding did not give good results, the glands were cut by hand into slices 0.1 — 0.3 mm. thick. With the above-mentioned prolonged treatment with the mounting solution before mounting, fully transparent preparations were obtained.

It was found that the thyroid gland, probably because of its exceptional richness in bloodvessels, was difficult to stain by this method, as compared with other organs, including smooth muscles and salivary glands, on which we also tested the method. The methylene-blue concentration must be appreciably lower when staining the nerves of the thyroid gland so as to avoid staining the thyroid cells. As, according to the



*Fig. 2.*

- A. Perivascular nerve plexus. After vagotomy.
  - B. Interfollicular branches from a more deeply lying perivascular nerveplexus. Normal animal.
  - C. Nerve fibres forming a well developed interfollicular network. Normal animal.
  - D. Interfollicular nervefibres. The follicles are seen as round well defined formations. Normal animal.
- All the sections from Schabadash stained thyroid gland.

literature, the colour of the stained nerve fades gradually, all the preparations were studied within a few days of mounting. A re-examination which took place about one year after the original experiment (and mounting), showed, however, that the initial colour had not changed.

A few experiments were made by the original method of placing the gland in a  $+37^{\circ}$  C. methylene-blue solution. Approximately twice as high a methylene-blue concentration was used. The conditions were otherwise the same as with injection staining. The larger nerve fibres stained extremely well, but the interfollicular network could not be seen with this method.

For the sake of comparison, staining with silver was also done by the usual methods: *Cajal*, *Gross-Schulze (Romeis)*, *Glees* (1946).

### *Nerve Operations.*

Vagotomy, sympathectomy and medullotomy were performed on guinea pigs in order to study the appearance and reactions of the thyroid gland after these operations.

Operation techniques: In vagotomy and sympathectomy a medial cut was made on the frontal side of the neck. The nerve-vessel cord was found on the right side, the nerve was dissected free, severed in the clavicular area and removed carefully in a cranial direction. By severing or giving a little jerk the ganglion nodosum was in most cases removed with the nerve. Sympathectomy proved technically more difficult as the sympathetics are finer, lie deeper and are often divided into several strands. In many cases it was possible, however, to remove the sympathetic nerves including the superior cervical ganglion. After the nerve operation, the isthmus of the thyroid gland was cut through.

In medullotomy the cut was made on the dorsal side of the neck. The soft parts were split open and the lower cervical vertebra (seventh cervical to first thoracic) were dissected free, and laminectomy and destruction of the medulla was then performed.

All operations were made under ether anaesthesia. Post-operative treatment included treatment with heat and oxygen tent. Before suturing some microcrystals of sulfathiazole were sprinkled into the wound. In some cases abscesses and hematomas were observed, but these animals have not been used in the experimental series. This includes, apart from the completely healed animals, only those that had trivial secondary inflammations or small hematomas.

At various times, 6—29 days after vagotomy and 4—21 days after sympathectomy, staining was done. Finally autopsy was performed under a lens to check the results of the operation.

According to *Glimstedt & Hillarp* (1942) and others the affinity of the autonomic nerves for methylene-blue disappears completely in warm-blooded animals about 48 hours after section of the nerve. According to *Lawrentjew & Borowskaja* (1936) fragmentation of the nerve fibres occurs before the staining ability disappears, as on staining with silver. At least a month is probably required for a complete autonomic regeneration (*Lee*, 1930).

#### *Trade preparations and thyrotrophin.*

The effect of Ergotamine tartrate (Sandoz) and Adrenaline (Parke Davis & Co.) on thyroid function has been studied. As a thyrotrophin we used Ambinon (Organon). These preparations were given intraperitoneally.

#### *Measurement of activity.*

Activity was measured partly by the ratio between the weight of the thyroid gland and the weight of the animals, but as this method is not reliable the main weight was placed on cell height in the microscopic picture. The method of measuring activity from the cell height has been studied closely by *Borell* and others (lit. see *Borell*, 1945).

The preparations were fixed in Susa (*Romeis*) for 24 hours, embedded in paraffin and sliced and stained by *Häggqvist's* iron hematoxylin method. The cell height was measured with



an ocular micrometer on a Zeiss microscope, 1000 x magnification. In each half of the gland, 25 or 50 follicles were measured on 2—4 planes through the gland. The marginal portions of the gland were not measured. In these preparations the general appearance of the thyroid gland was also studied.

The investigation material consisted of about 150 guinea pigs of all ages.

## RESULTS

### *The morphology of the nerves of the normal thyroid gland.*

By the use of the above-described staining with methylene-blue we could differentiate the following structures in agreement with *Nonidez* and others.

1) A perivascular plexus. 2) A perifollicular plexus, partly branching off from the perivascular, partly originating in the nerve-fibres which crossed the capsule or entered via the hilus. 3) Larger nerve-fibres, many of which contained myelin. These reached the parenchyma partly via the hilus and partly by breaking directly through the capsule. These larger fibres seemed to pass for long distances through the parenchyma with no branchings, except for one or two here and there. In no case did we observe these fibres splitting into finer networks or connecting with any such networks.

The perifollicular network was very fine and surrounded the follicles with relatively close meshes. No endings, such as those described by *Popow* (1927), *Rossi & Lanti* (1934/35) and by others, were observed. In several cases, however, we observed structures which resembled *Cajal's* interstitial cells lying parafollicularly. Owing to the difficulties in staining the nerves of the thyroid gland these delicate structures were seen only sporadically and we did not attempt to analyze them.

Despite experimental staining with silver and methylene-blue of the thyroid glands of new-born and older guinea pigs, we never saw any ganglion cells, such as those described by *Nonidez* (1931 a) in thyroid glands of dogs.

*Morphology after section of the nerves.*

Staining with methylene-blue after vagotomy showed the same nerve structures as in normal animals, both quantitatively and qualitatively. After sympathectomy, however, a large number of nerve-fibres disappeared from the histological picture (see Table 1). In certain places granular and fragmentary structures were seen, which some authors claim to be degeneration products. Whether these changes are due to nerve degeneration or whether they are purely staining artefacts cannot be ascertained. The above-mentioned nerve fibres (1, 2, 3) disappear about equally after the operation. Possibly the perivascular plexus is affected less than other elements.

No degeneration of thyroid cells was seen after either vagotomy or sympathectomy. Nor was the height of the cell affected as seen in Table 1. A cell height of just about  $9\ \mu$  must be accepted as lying within the normal variation. *Borell* claims this to be  $7.5 - 9.6\ \mu$ .

That the staining process in itself had no effect on the cell height is shown by the fact that sympathectomized controls (6 animals) in which no staining had been performed had a mean cell height of  $8.7\ \mu$ . The weight of the thyroid gland, however, may be expected to change on staining as various amounts of fluid remain in the organs after the injection. For this reason no thyroid weights have been included.

For the sake of clarity the results have been tabulated. This type of table must, naturally, be very schematic, but the main characteristics are evident.

As seen from the table, at autopsy the operation was found to have been incomplete in seven of the sympathectomized animals (in only two of which, however, the cranial ganglia remained). The difficulty in performing a total sympathectomy has also been pointed out by *Tronconi* (1937) and others. The absence of macroscopically observable vagus or sympathetic branches at autopsy is, naturally, no evidence of total denervation. Autonomic fibres may run e. g. with the vessels or be so fine as to escape notice. It is possible that the nervous structures observed in the microscopic preparation after sympathec-

tomy, are derived from fibres which have been overlooked at the operation.

*Glandular activity after nerve extirpation and thyrotrophin stimulation.*

In order to obtain an idea of the reactions of the thyroid gland after denervation, thyrotrophin was injected into animals a week after they had undergone right-sided sympathectomy, with which, as described above, large portions of the thyroid nerves were removed. The thyrotrophin dose (one unit

*Table 1.*

Fragmented and granulated structures = (+)      Moderate number = ++  
 Small number of observed nerves = +      Very large number = ++++  
 Large number = +++

*Right-sided vagotomy:*

Animal No.	Number of days after op.	R. lobe nr. of nerves	R. lobe cell height in $\mu$ .	L. lobe nr. of nerves	L. lobe cell height in $\mu$ .	Autopsy
890	5	++	$9.1 \pm 0.07$	++	$8.7 \pm 0.21$	Healing good. Remaining vagus fibres 0.
882	8	+++	$8.6 \pm 0.25$	+++	$8.0 \pm 0.21$	Hematoma in the scar. Remaining vagus fibres 0.
914	10	+	$9.4 \pm 0.08$	+	$9.5 \pm 0.13$	Healing good. Remaining vagus fibres 0.*)
915	10	+++	$9.1 \pm 0.12$	++	$9.2 \pm 0.11$	Healing good. Remaining vagus fibres 0.
884	14	+	$8.4 \pm 0.27$	+	$6.9 \pm 0.22$	Small hematoma in the fibres 0.
892	27	++	$9.4 \pm 0.10$	+	$9.6 \pm 0.13$	Healing good. Remaining vagus fibres 0.
901	28	+	$9.7 \pm 0.14$	++	$9.6 \pm 0.15$	scar. Remaining vagus fibres 0.
903	29	++	$8.6 \pm 0.13$	+++	$10.4 \pm 0.11$	Healing good. Remaining vagus fibres 0.
8 animals	16+		$9.0 \pm 0.16$	15+	$9.0 \pm 0.40$	*) Left-sided vagotomy.

Table 1 continued.

*Right-sided sympathectomy.*

Animal No.	Number of days after op.	R. lobe nr. of nerves	R. lobe cell height in $\mu$ .	L. lobe nr. of nerves	L. lobe cell height in $\mu$ .	Autopsy
933	4	+	$9.9 \pm 0.30$	—	$8.7 \pm 0.21$	Healing good. Remaining symp. fibres 1.
969	4	(+)	$9.7 \pm 0.35$	++	$10.0 \pm 0.27$	Healing good. Remaining symp. fibres 0.
927	7	+	$9.0 \pm 0.30$	+++	$7.4 \pm 0.33$	Sec. inf. Remaining symp. fibres 1.
965	9	(+)	$8.7 \pm 0.21$	++	$10.2 \pm 0.23$	Healing good. Remaining symp. fibres 0.
928	11	+	$7.7 \pm 0.11$	+++	$9.3 \pm 0.21$	Hematoma. Remaining symp. fibres 1.
936	12	+	$9.2 \pm 0.23$	+++	$7.4 \pm 0.33$	Healing good. Remaining symp. fibres 1.
938	12	+	$8.2 \pm 0.27$	+	$7.9 \pm 0.15$	Healing good. Cran. gangl. remaining.
956	13	(+)	$9.6 \pm 0.35$	++++	$9.7 \pm 0.37$	Healing good. Remaining symp. fibres 1.
935	14	+	$9.3 \pm 0.17$	+	$9.3 \pm 0.20$	Healing good. Remaining symp. fibres 0.
967	21	(+)	$8.4 \pm 0.38$	+++	$8.5 \pm 0.27$	Healing good. Cran. gangl. remaining.
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10 animals	6+4(+)		$9.0 \pm 0.23$	22+	$8.8 \pm 0.33$	

per 100 grams of body weight for one or two days) should have caused, according to *Borell* a distinct increase in the height of the cell 24 hours after the last injection.

As *Lowe, Ivy & Brock* (1945) have shown that thyrotrophin and exposure to cold have, on the whole, a similar effect on the metabolic rate in bilaterally sympathectomized and in normal animals, we were satisfied with a small series of four animals. We simply wished to find out whether the results were the same with one-sided sympathectomy, serving as an indicator of activity.

The mean cell height in the fo operated side and 12.0 on the other side was a significant difference.

*Gland activity after medullotomy and thyrotrophin stimulation.*

Some authors, Borell (1945) and others have found thyrotrophin containing substance in the cerebrospinal fluid, the diencephalon and the choroid plexus. It has been suggested that thyrotrophin could pass up the stalk of the hypophysis and affect higher centres. As a result these centres would discharge stronger vegetative impulses which would travel down the cervicospinal cord.

In order to interrupt this mechanism we performed medullotomy in the lower cervicospinal cord in guinea pigs, which were then given injections of thyrotrophin (2 — 3 units per 100 gm. of body weight) and killed after 24 hours. Five animals treated in this manner showed a cell height of  $9.6 \pm 0.57 \mu$ . In ten controls the cell height was  $9.5 \pm 0.44 \mu$  and t-analysis showed no difference between these and the experimental animals.

The small effect of thyrotrophin in this experiment was probably due partly to the relatively high room temperature during the experiment, which causes a decrease of the cell height and partly to the relatively large-sized animals used. As an unmistakable thyrotrophic effect, however, was observed histologically, and as this effect was as marked in the operated animals as in the controls, we venture to maintain, on the basis of this experiment, that medullotomy in the lower cervicospinal cord does not change the reaction of the thyroid gland to thyrotrophin. This disproves the theory that thyrotrophin has a central point of attack.

*Gland activity on treatment with adrenaline and ergotamine.*

As the innervation of the thyroid gland was found to consist principally of sympathetic elements, we were tempted to test the effect of sympathomimetic substances (adrenaline) and sympatholytics (ergotamine). In order to demonstrate more readily the variations in the cell height, the animals used in the adrenaline test were placed in a warm atmosphere of

+ 24° C. to + 30° C. for two—three weeks, which reduces the cell height to a minimum and the animals used in the ergotamine test were placed in a cold atmosphere of + 4° C. to + 6° C. which increases the cell height, for one—two weeks before and during each experiment (*Borell & Holmgren, 1943, Borell, 1945*).

The adrenaline was injected in doses of 0.10 — 0.25 mg. twice daily for the last three-seven days of the warm period. No increase of the cell height was observed after these injections, the average cell height in nine treated animals was  $7.5 \pm 0.37 \mu$ , in eight normal animals  $8.2 \pm 0.23 \mu$ . On the contrary, a slight decrease was noticed, but this was not significant ( $p < 0.1$ ).

The ergotamine was injected in doses of 1—2 mg. twice daily for the last four—seven days of the cold period. See Table 2.

As seen from the table, the cell height, which was increased by cold, decreases on ergotamine administration from  $12.6 \pm 0.27 \mu$ . to  $10.0 \pm 0.44 \mu$ . The difference is statistically significant ( $p < 0.001$ ). There is also a fall in the relative weight of the thyroid gland, but this figure shows greater variation and is undoubtedly less reliable as an indicator of thyroid activity than the cell height.

Table 3 shows the effect of ergotamine in animals treated with thyrotrophin. These animals were kept at room temperature (+ 16—18° C.). The thyrotrophin dose was the same as in *Borell's* experiment (1945) and should, according to this author, have produced a cell height of about 13.0  $\mu$ .

As seen from Table 3, ergotamine reduces the effect of thyrotrophin. In animals treated with thyrotrophin the cell height is  $12.5 \pm 0.32 \mu$ , whereas in animals treated with both thyrotrophin and ergotamine it is  $11.4 \pm 0.22 \mu$ . The difference, however, is not quite significant ( $p < 0.02$ ).

In our experiments we obtained an increase in the cell height caused by cold or thyrotrophin, which in both cases amounts to about 4  $\mu$  above the normal cell height of 8—9  $\mu$ . Ergotamine reduced the increase of the cell height caused by

Table 2.

Animals treated with cold.

Weight of animal in gm.	Weight of thyroid gl. in mg. per 100 gm. of body weight	Cell height in $\mu$ .	
240	26.3	$12.3 \pm 0.17$	Cold 14 days
257	23.3	$12.6 \pm 0.22$	» » »
266	18.8	$13.3 \pm 0.19$	» » »
337	19.6	$12.1 \pm 0.13$	Cold 21 days
390	11.8	$12.5 \pm 0.16$	» » »
354	16.7	$12.2 \pm 0.20$	» » »
400	22.5	$14.1 \pm 0.20$	» » »
414	18.1	$11.7 \pm 0.30$	» » »
8 animals	19.5	$12.6 \pm 0.27$	

Animals treated with cold and ergotamine (Gynergen).

Weight of animal in gm.	Weight of thyroid gl. in mg. per 100 gm. of body weight	mg. of ergotamine	Cell height in $\mu$ .	
300	19.3	7	$8.3 \pm 0.15$	Cold 8 days. 1 mg. of erg. $\times 2$ for 4 days.
320	15.3	13	$11.9 \pm 0.21$	Cold 14 days. 1 mg. of erg. $\times 2$ for 7 days.
293	13.7	13	$12.0 \pm 0.23$	— » —
308	23.4	13	$7.4 \pm 0.20$	— » —
298	22.1	13	$7.1 \pm 0.21$	— » —
197	17.8	13	$9.9 \pm 0.16$	— » —
348	14.9	13	$11.0 \pm 0.19$	Cold 21 days. 1 mg. of erg. $\times 2$ for 7 days.
364	18.7	13	$11.3 \pm 0.16$	— » —
380	16.6	13	$11.4 \pm 0.15$	— » —
425	20.0	13	$9.5 \pm 0.17$	— » —
317	20.5	13	$11.8 \pm 0.16$	— » —
312	13.8	14	$8.9 \pm 0.15$	Cold 14 days. 2 mg. of erg. $\times 2$ for 4 days.
285	29.5	14	$10.1 \pm 0.14$	— » —
325	14.8	14	$9.1 \pm 0.15$	— » —
14 animals	18.0		$10.0 \pm 0.44$	

cold to about  $1.5 \mu$  and the increase caused by thyrotrophin to about  $3 \mu$ . Thus ergotamine reduces the cell height-increasing ability of cold and thyrotrophin by about 60 and 25 per cent, respectively.

Table 3.

Animals treated with thyrotrophin (Ambinon).

Weight of animal in gm.	Weight of thyroid gl. in mg. per 100 gm. of body weight	Cell height in $\mu$ .	
310	15.2	$13.1 \pm 0.17$	2 units Ambinon daily for 3 days.
290	15.9	$12.4 \pm 0.20$	— » —
390	16.2	$13.0 \pm 0.27$	— » —
235	17.9	$11.3 \pm 0.23$	— » —
350	15.4	$12.5 \pm 0.16$	— » —
5 animals	16.1	$12.5 \pm 0.16$	

Animals treated with thyrotrophin and ergotamine.

			Ergot. in mg.	
280	15.4	$11.9 \pm 0.14$	8	Ergot. 1 mg. $\times$ 2 for 4 days.
330	15.5	$11.2 \pm 0.16$	8	2 units Ambinon daily for 3 days.
280	13.2	$10.7 \pm 0.18$	8	— » —
320	14.1	$11.8 \pm 0.23$	8	— » —
280	12.9	$11.3 \pm 0.23$	8	— » —
5 animals	14.2	$11.4 \pm 0.22$		

## DISCUSSION

As our experiments with vagotomy and sympathectomy show that the nerves of the thyroid gland do not seem to be affected by vagotomy but that large portions of the nerve-net



are changed or disappear on sympathectomy, we maintain that most of the gland nerves originate from the sympathetic system. *Flatow & Schilf* (1928) and others, also hold that there is no evidence of a parasympathetic innervation. *Nonidez* (1931 b) considers that the myelinated nerve-fibres which may be observed, arise from the superior laryngeal nerve. We do not agree with this opinion, however, as in our experiments the myelinated nerve-fibres have disappeared completely or partly from the microscopic picture after sympathectomy.

A number of authors, *Tronconi* (1937) among others, claim that a degeneration of the parenchyma occurs after sympathectomy. *Tronconi* also emphasizes the difficulty of performing a total sympathectomy. We are also convinced of this difficulty. But even in those cases where staining with methylene-blue showed marked reduction in the number of nerve-fibres in the gland, and post-operative autopsy showed macroscopically total absence of sympathetic nerve-fibres, we have found no signs of degeneration or hypoactivity of the thyroid parenchyma on the operated side.

*Uotila* (1939) maintains that a temporary, bilateral, moderate reduction of the cell height is produced on unilateral sympathectomy. We have not obtained this result in our experiments, but they are, admittedly not suitable for this kind of investigation, and we do not wish to dispute *Uotila's* results. *Uotila*, however, used rats in his experiments, and we do not consider that the thyroid glands of these animals which even normally are irregularly active, are suitable for determining the cell height.

Neurohistologic findings on thyroid innervation have been interpreted according to the general conceptions of the peripheral innervation mechanism in the autonomic nervous system. *Stöhr*, (Lit. see: *Hillarp*, 1946), *Sunder-Plassmann* (1941) and others, assert that the effector cell is diffusely interwoven with the nervous elements, the so-called terminal reticulum. *De Castro*, *Lawrentjew*, *Nonidez* (lit. see *Hillarp*, 1946) and others, consider that the effector cell is innervated individually with free or intraprotoplasmatic nerve-endings.

*Boeke* (1940) holds that the autonomic peripheral innervation structure consists of a syncytical network of Schwann's cells in which communicating neuro-fibrils connect continuously with the effector cell. By means of the silver staining methods used in earlier investigations it is at present almost impossible to decide critically which school is based on the most convincing facts.

*Hillarp* (1946) has made a close test of Ehrlich's method of staining nerve-endings with methylene-blue and has found that the method may be modified according to each organ, so that even the finest nervous structures may be studied. He found that the staining is specific for nervous substance and that it gives an adequate picture of the vital conditions. Moreover, it is possible to study the degeneration of the nerves, provided the methylene-blue-concentration used is not too high. As already mentioned, staining of the thyroid glands with methylene-blue, however, had one disadvantage, in that it was liable to overstain the parenchyma of the thyroid gland which is extremely rich in vessels, and thus disguise or render indistinct any possible finer structures. The interfollicular network, however, was always distinctly observable but no nerve-endings or connections between network and parenchyma cells could be demonstrated.

As we failed to determine, histologically, whether connections between nerves and thyroid cells exist, we attempted to find the answer along more physiologic lines. *Cannon's* (1914, 1916, 1922), *Haney's* (1932) and *Uotila's* (1939, 1940) observations are mentioned. Our experiments with one-sided sympathectomy gave, on the whole, no effect on the cell height and lead to the same conclusion as *Lowe, Ivy & Brock's* (1945) i. e. in showing that thyrotrophin affects the thyroid cell independently of the sympathetic innervation of the thyroid gland. This view is also supported by experiments made by *Eger & Titze* (1943), who produced adequate thyrotrophin action on a transplanted thyroid gland, and by *Eitel, Krebs & Loeser* (1933) who obtained adequate thyrotrophin action on thyroid tissue in vitro. Thus there are many arguments in favour of

the opinion that the thyroid gland on the whole functions normally when denervated.

It may be concluded that our studies have not furnished any evidence of a secretory innervation of the thyroid gland and that the interfollicular network which may be observed in the gland consisted merely of fragments of the vascular nerves.

*Nonidez* (1931 a) suggests that the gland is regulated indirectly via the vessels. He has advanced the hypothesis that the ganglion cells in dogs, described by him, would serve as a kind of receptor organs which register the width of the vessels. These organs would discharge impulses which would travel along the myelinated fibres via the superior laryngeal nerve cranically, and in the central nervous system be switched over to efferent nerves. We have not found any ganglion cells in guinea pigs, despite a close study of glands from some 50 animals of all ages stained with methylene-blue and silver, and the myelinated fibres seem to us to be of a sympathetic origin. Therefore, we do not consider that the *Nonidez*' theory, on the whole, is applicable to all animals.

As the thyroid gland appeared to be innervated principally by sympathetic nerves, and as these, according to the literature, also act on the hypophysis, we attempted to test the effect of adrenaline and ergotamine. The effect of adrenaline on the thyroid gland has already been studied by several investigators. *Issekutz & Harangazo-Oroszy* (1942) found that adrenaline caused a rise in the metabolic rate. In our experiments adrenaline produced a slight decrease of the cell height, which, however, lies within the limits of statistical error. Ergotamine, on the other hand, reduced the cell height-increasing effect of cold and thyrotrophin by about 60 and 25 per cent, respectively. It should here be mentioned that ergotamine, according to several investigators, has an antithyroid effect. This opinion was held by *Schimert* (1942) who, on the basis of clinical experiments, reports that ergotamine reduces the effect of the administered thyroid substance.

The question then arises, what is the site of action of ergo-

tamine in our experiments? Does ergotamine check the activating impulses in the hypophysis, or does it act on the vessels? As our own and other authors' investigations show that there is no secretory innervation of the thyroid gland, it seems probable that ergotamine acts by checking the impulses to the hypophysis and by affecting the vessels. The difference in degree between the reduced effect of cold and that of thyrotrophin may support this view. On exposure to cold it may be possible both that the impulses to the hypophysis are checked by ergotamine and that the smaller amounts of secreted thyrotrophin are prevented by the ergotamine action on the vessels from reaching the thyroid cells. On administration of thyrotrophin plus ergotamine, on the other hand, ergotamine would inhibit the effect of thyrotrophin only by its action on the vessels. There are, however, other possible interpretation, e. g. a certain genuine antagonism between thyrotrophin and ergotamine.

### SUMMARY

1. The nerves of the thyroid glands in guinea pigs have been studied histologically, principally by means of staining with methylene-blue. The following structures were observed:
  - a. Perivascular plexus.
  - b. Interfollicular plexus.
  - c. Myelin-containing nerve-fibres, running through the parenchyma more directly.
2. Most of the observed structures have been found to consist of sympathetic elements.
3. No direct contact or connection between nervous system and thyroid epithelium has been observed, either on histological or physiological examinations. The gland appears to react adequately and almost independently of its nerves.
4. No evidence has been obtained in support of the theory that thyrotrophin has a central point of attack.

5. Administration of ergotamine reduced the cell height-increasing effect of cold and thyrotrophin by about 60 and 25 per cent, respectively.

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## LE CYCLE VAGINAL AU COURS DU DIABETE ALLOXANIQUE CHEZ LE RAT

PAR

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L'introduction de l'alloxane au service de la biologie expérimentale a contribué essentiellement aux recherches concernant le syndrome du diabète sucré. Depuis qu'on a constaté que cette matière produit chez certaines animaux de laboratoire, comme le rat et le lapin un état diabétique très analogue au diabète sucré humain à savoir hyperglycémie, glycosurie, polyurie, polydipsie, acetonurie et lipémie (*Jacobs*, 1937), alloxane a été employé dans ce but par de nombreuses investigateurs. Nous savons maintenant que ce corps provoque une nécrose sélective dans les îlots de Langerhans et que le diabète résultant est d'origine pancréatique. (*Goldner & Gomorri*, 1947). Il faut d'autre part noter l'observation intéressante de *Walpole et Innes*, vérifié par *Adams* (1949), que l'atrophie pancréatique acineuse chez le chien, provoqué par une ligature des canaux pancréatiques, rend l'animal réfractaire à l'alloxane. Ceci semble néanmoins indiquer la participation du tissu acineux au mécanisme diabetogène de l'alloxane. En outre nous savons que le diabète ainsi provoqué peut être temporaire ou permanent suivant le dosage de la matière diabétogène (*Davis, Fugo & Lawrence*, 1947). La plupart des chercheurs ont aussi constaté que les dommages des différents organes sont plus

marqués dans la période aigue, surtout pendant la première semaine, que plus tard.

Sur le plan expérimental *Pratt* a pu noter chez la chienne artificiellement diabétique la cessation du cycle génital. *Davis et al.* (1947) avaient travaillé avec des rats diabétisés par l'alloxane. Ils ont suivi chez six animaux le cycle vaginal et écrivent ce que suit: »Following the hyperglycemia the oestrous pattern changed. Oestrous smears occurred with considerable irregularity and the interval was prolonged. Normal 4—5 day pattern was replaced by 9—12 days and the animal remained in dioestrus the greater part of each cycle.«

Notre intention était d'aborder la question des relations entre l'activité insulaire pancréatique et le fonctionnement des ovaires. Depuis très longtemps on sait que ces liaisons sont intimes (*Vogt*, 1927). On avait noté les irrégularités, surtout les aménorrhées, et la stérilité concomittante au diabète sucré et la notion d'une ménopause précoce des femmes diabétiques figurait dans les traités de gynécologie. Après la découverte de l'insuline on pouvait encore constater que ces dérangements cédaient au moins partiellement à la thérapie insulinique adéquate, qui donnait une preuve étiologique concluante.

## LA REALISATION DU TRAVAIL

L'animal-cobaye était le rat gris d'une souche cultivé depuis des années au laboratoire scientifique universitaire. L'âge des rats était environ trois mois. Le matériel étudié se porte sur vingt cas, dont quatre sont restés comme animaux de contrôle. Avant de commencer l'expérience nous avons observé les rates pendant au moins 3—4 semaines en prenant des frottis vaginaux quotidiennement. Leur régime resta inchangé au cours du travail et contient suffisamment de vitamines. Pendant ce temps-la leur taux du sucre sanguin fut vérifié. Cette manipulation fut pratiqué par une laborantine expérimentée, suivant la méthode de *Hagedorn-Jensen*, une méthode universellement acceptée comme digne de confiance. Le poids initial des animaux fut naturellement aussi noté. Le cycle



vaginal des animaux intacts était régulier à  $4 \pm 1$  jours, leur poids variait entre 200—300 grammes et le sucre sanguin s'aurait être en moyen 0,110 mg % (min. 0,088 et max. 0,142).

L'expérience à proprement parler commença par une piqûre intramusculaire d'alloxane dosé de 90 à 150 mg par kilo du poids. Le temps d'observation varia de trois jours à huit semaines. Pendant cette période la prise des frottis vaginaux fut continué régulièrement et l'évaluation du sucre sanguin aussi bien que le pesage furent répétés au moins une fois par semaine. Si l'état général des animaux devenait grave et un coma était imminent nous donnions un peu d'insuline pour les remonter afin de prolonger le temps de l'expérience et d'autre part pour pouvoir suivre l'effet de cette drogue spécifique. Le tableau N:o 1 vous montre l'ensemble de ce travail.

Tableau N:o 1.

N:o	Durée d'observ. en semaine	Alloxane mg/kg	Sucre sanguin		Perte de poids %	Coma	Insuline
			Norm.	Max.			
1	8	100	0,120	0,516	26	—	+
2	»	150	0,116	0,588	20,6	+	+
3	6	»	0,110	0,618	36	+	+
4	»	100	0,118	0,572	22	—	+
5	4	»	0,106	0,124	10	—	—
6	»	»	0,114	0,130	8	—	—
7	»	»	0,118	0,124	7	—	—
8	»	»	0,111	0,532	12	—	+
9	3	»	0,094	0,214	11	—	—
10	»	»	0,088	0,342	28	—	—
11	»	»	0,110	0,356	13	—	—
12	2½	»	0,098	0,416	40	+	—
13	»	»	0,088	0,642	38	+	+
14	1	»	0,119	0,406	—	+	+
15	½	150	0,106	—	—	—	—
16	»	90	0,105	0,662	—	+	+
17	8	0	0,142	—	0	—	—
18	»	»	0,124	—	»	—	—
19	»	»	0,112	—	»	—	—
20	»	»	0,122	—	»	—	—

Les animaux  
de contrôle

Le plan général du travail.

## LE MATERIEL ETUDIE

Comme on voit d'après le diagramme N:o 1 les quatre animaux de contrôle ont eu un cycle vaginal régulier et aucun d'entre eux n'a perdu de poids. L'état général a aussi été sans reproche. Parceque le comportement des rates diabétiques a été assez variable nous jugerons utile de discuter un peu chaque cas à part. Nous les présentons en ordre chronologique suivant le temps d'observation. Le diagramme N:o 1 illustre le cycle vaginal de chaque rate.

Cas N:o 1. Le temps d'observation était huit semaines. Cet animal a reçu 100 mg d'alloxane par kg/poids et développa un syndrome diabétique caractéristique; les valeurs du sucre sanguin étaient: 0,120 mg/% (le taux initial), 0,316 — 0,382 — 0,452 — 0,356 — 0,516 — 0,510 — 0,426 — 0,470. Au commencement la rate était en très bonne condition, prenant même de l'embonpoint, mais durant la 4 à 6 semaine de l'expérience elle se portait moins bien et recut de 1 à 3 unités d'insuline par jour. Pendant les deux dernières semaines l'état se stabilisa et elle n'avait plus besoin d'insuline. Dans les huit semaines elle avait perdu 26 % de son poids initial. En tout elle présentait trois fois les 3:ème, 17:ème et 32:ème jours une kératinisation vaginale. Ce phénomène se produisit malgré les valeurs relativement hautes du sucre sanguin et les premières fois sans intervention insulinique.

Cas N:o 2. Le temps d'observation était aussi huit semaines, mais l'animal a reçu 150 mg d'alloxane par kg/poids. Ce cas est assez analogue au précédant quoique faisant un diabète plus grave. La période critique commença aussi vers la quatrième semaine ou l'animal fut une fois dans un état comateux et recut depuis presque continuellement de l'insuline de 1½ à 3 unités par jour. Les valeurs du sucre sanguin furent: 0,116 (initiale) 0,196 — 0,352 — 0,392 — 0,556 — 0,588 — 0,496 et 0,568 mg/%. La perte du poids fut 20,6 % pendant les huit semaines. Les jours de kératinisation vaginale furent: 3—4, 6—8, 12—13, 18—19, 23, 34—35, 38, 40 et le 53:ème et 54:ème jour. Ici nous pouvons de nouveau constater le prolongement de la période diestrals mais néanmoins l'apparition des kéra-

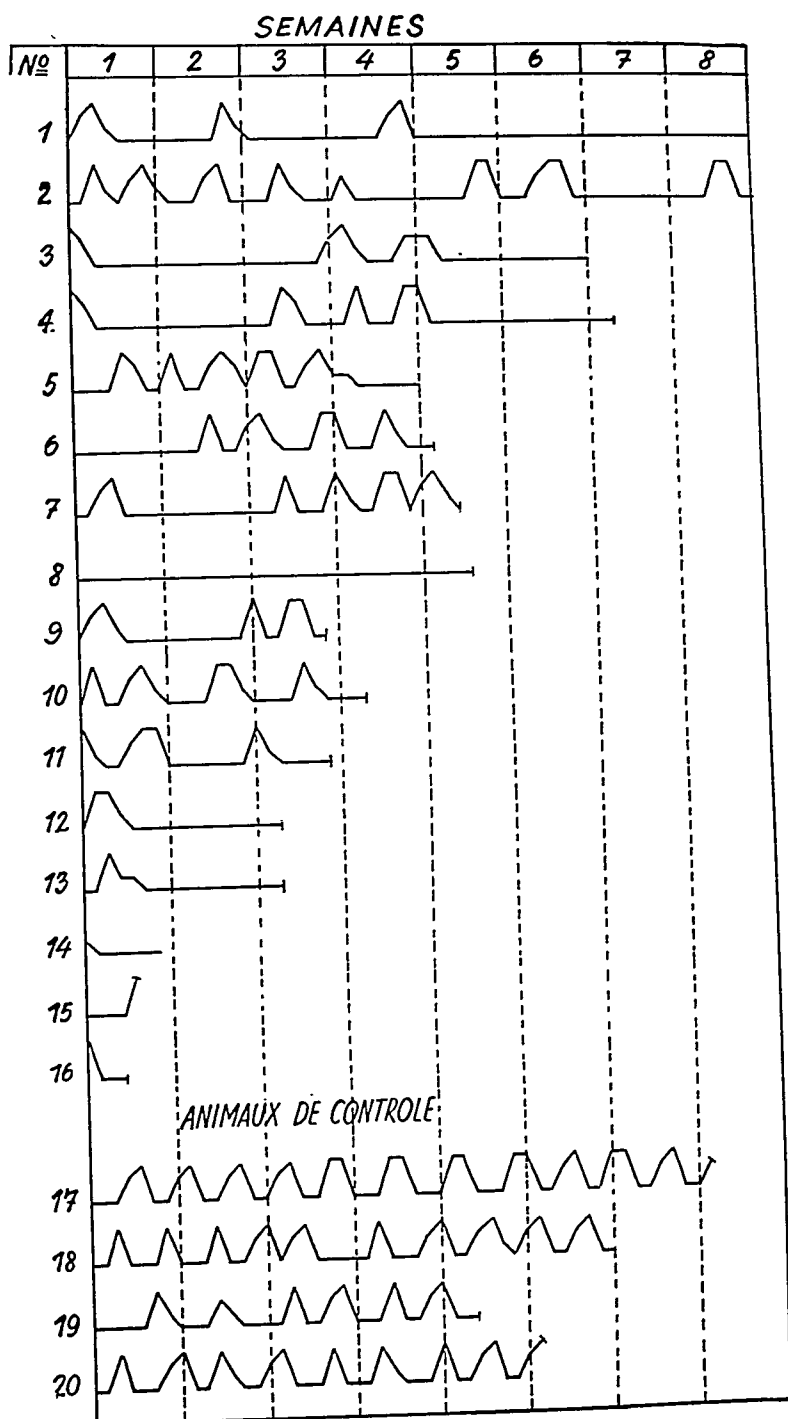


DIAGRAMME N:O 1.  
Le cycle vaginal des rates.

tinisations malgré les taux extrêmement élevés du sucre sanguin. L'état comateux causait visiblement un intervalle de dix jours. (Diagramme N:o 2.)

Cas N:o 3. Le temps d'observation fut six semaines et l'animal reçut 150 mg d'alloxane par kg/poids. La rate fit un diabète très grave faisant un coma diabétique deux fois. Elle a reçu des doses massives d'insuline jusqu'à  $5\frac{1}{2}$  unités par vingt-quatre heures à partir du 8:ème jour. Les valeurs du sucre sanguin furent: 0,110 (initiale), 0,098 — 0,618 — 0,452 — 0,418 — 0,586 mg/%. Ici on peut constater une courte période d'hypoglycémie initiale, ce qui a d'ailleurs déjà été noté par *Jacobs*. L'animal ne présenta que deux fois une kératinisation vaginale à savoir les 21 — 23 et 27 — 29 jours depuis le commencement de l'expérience. La kératinisation semble ici être le résultat d'un traitement insulinique intensif. Après l'interruption de ce traitement l'animal mourut dans une semaine en coma diabétique. La perte du poids fut 36 %.

Cas N:o 4. Le temps d'observation fut six semaines et l'animal reçut 100 mg d'alloxane par kg/poids. Ce cas est assez analogue au précédent mais un peu moins grave et sans d'attaques comateuses. Le taux du sucre sanguin fut: 0,118 (initial), 0,356 — 0,566 — 0,456 — 0,572 — 0,512 — 0,450 — 0,474 mg/%. Le dosage d'insuline était aussi moindre variant entre une et trois unités par jour. La kératinisation apparut les 16—17 le 23 et les 27—28 jour. L'état général de cette rate s'affaiblit continuellement et le 46:ème jour de l'expérience elle fut tuée. La perte de poids était 22 %.

Cas N:o 5—8. Puis nous avons quatre cas dont le temps d'observation était quatre semaines et qui reçurent tout les quatre 100 mg d'alloxane par kg/poids. La dernière de ces rates (N:o 8) réagit relativement fort en faisant un diabète assez sévère, tandisque chez les trois autres le taux du sucre sanguin resta presque au niveau normal. Le cas N:o 8 est intéressant parceque l'animal resta pendant tout le temps d'observation sans kératinisation vaginale malgré un état magnifique et malgré des piqûres d'insuline de une à trois unités par jour pendant deux semaines. La perte de poids de cet animal fut

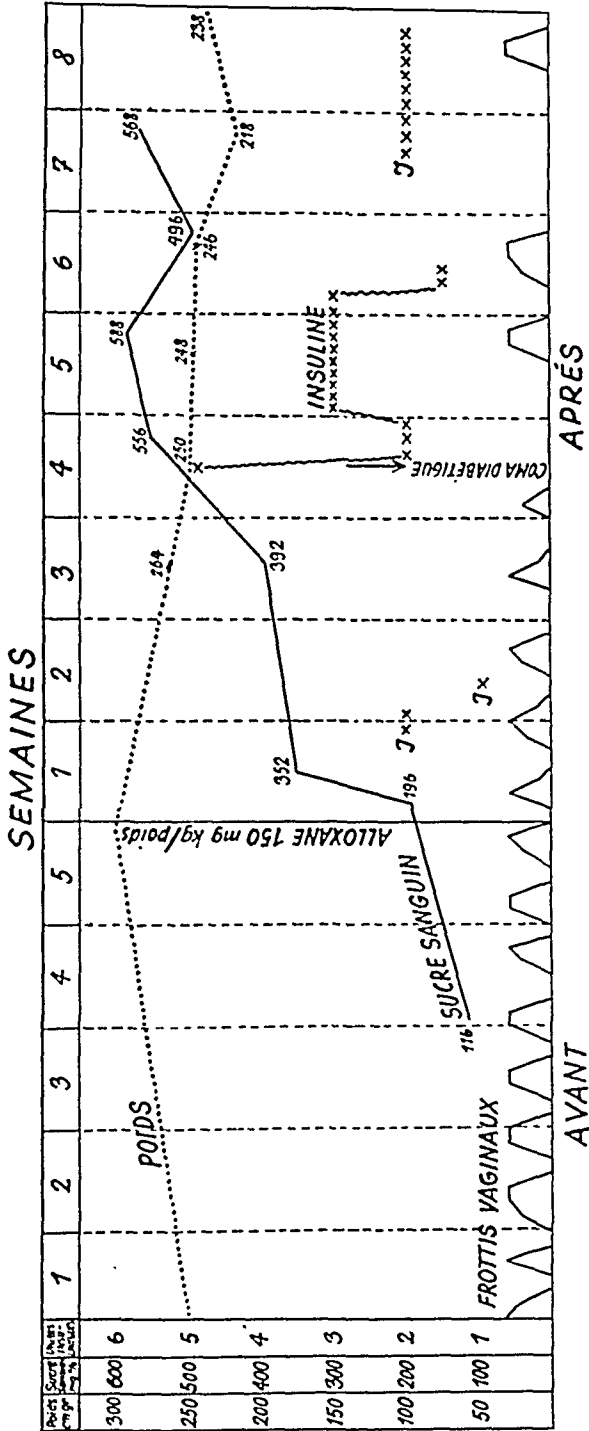


DIAGRAMME N:O 2.

Poids, sucre sanguin, frottis vaginaux et traitement dans cas N:O 2.

12 % et les valeurs du sucre sanguin furent: 0,111 — 0,532 — 0,374 — 0,528 — 0,490 — 0,438 mg/%. Dans les trois autres cas de ce groupe l'effet de l'alloxane fut insignifiant, les taux maxima du sucre sanguin étant 0,124, 0,130 et 0,124. Dans tous ces cas le cycle vaginal resta relativement inchangé, sauf une période de 7 à 12 jours au commencement. Ce phénomène et d'autre part une légère perte de poids resp. 10 %, 85 % et 7 % témoignent probablement donc d'une légère intoxication alloxanique. Bien entendu pas d'usage d'insuline.

Cas N:o 9—11. Le temps d'observation fut à peu près trois semaines et toutes les rates recurent 100 mg d'alloxane par kg/poids. Les trois animaux firent un diabète assez discrète étant donné qu'aucun n'avait besoin d'une thérapie insulinaire. Les résultats du dosage du sucre sanguin furent: cas N:o 9 0,094 — 0,214 — 0,110 — 0,134; cas N:o 10 0,088 — 0,330 — 0,296 et 0,342 et cas N:o 11 0,110 — 0,356 — 0,210 mg/%. Aucun présentait une tendance comateuse et le cycle vaginal était périodique mais à phases diestrales prolongées de 5 — 8 jours. Sans aucun raison visible la perte de poids était le double dans le cas N:o 10 en comparaison avec les deux autres à savoir 11 %, 28 % et 13 %. Est-ce que la sensibilité individuelle vers la toxicité alloxanique puisse varier tellement étant donné que l'effet diabétogène de ce corps était à peu près pareil dans les trois cas?

Cas N:o 12—13. Le temps d'observation fut 2½ semaines. Les deux rates présentaient un diabète grave après avoir reçu 100 mg d'alloxane par kg/poids. L'insuline fut employé seulement pendant quelques jours pour parer la première montée du sucre sanguin, mais nous avons après laissé la maladie se développer librement afin d'avoir des exemples de formes foudroyantes. Les deux cas ont abouti en dix-sept jours à un état comateux et furent ensuite abattus sans plus être drogués. Les valeurs du sucre sanguin furent: 0,088 — 0,642 — 0,446 et 0,098 — 0,414 — 0,416. La perte de poids fut chez les deux animaux catastrophique c'est à dire 40 % et 38 % en dix-sept jours. Les deux animaux furent à partir de 5:ème resp. 6:ème

jour sans kératinisation vaginale justement à l'époque où la toxicose diabétique commençait à s'imposer.

Cas N:o 14. est assez analogue aux précédents, mais l'animal ne put résister au premier choque diabétique et mourut spontanément dans un profond coma au bout d'une semaine. Les taux du sucre sanguin étaient: 0,119 — 0,228 et 0,406 mg/%. La dernière valeur a été noté le quatrième jour de l'expérience. Pendant cette dernière semaine de sa vie cette rate ne fit pas de kératinisation vaginale. La dose d'alloxane était 100 mg par kg/poids.

Les deux derniers cas sont assez démonstratifs au point de vue de la sensibilité différente à l'alloxane, quoique le temps d'observation n'excédait pas trois jours. La rate N:o 15 pesait 262 grammes reçut 150 mg d'alloxane par kg/poids et parut être au troisième jour, quand elle fut abattue, complètement réfractaire à l'alloxane (Le contrôle du taux du sucre sanguin a malheureusement échoué). La rate N:o 16 par contre pesant 240 grammes, recut seulement 90 mg d'alloxane par kg/poids et fut au bout de trois jours dans un état misérable profondément comateuse et le taux du sucre sanguin fut au 0,662 mg/%.

## LES RECHERCHES HISTOLOGIQUES

Notre intérêt se porta aussi sur l'aspect histologique de l'ovaire et du pancréas de nos animaux et l'autopsie fut pratiquée immédiatement après la mort des rats. Les organes furent conservés et plus tard étudiés microscopiquement. La coloration l'ovaire fut faite selon la méthode classique de van Giesson. Dans le pancréas par contre il était important de distinguer les différents catégories de cellules et les coupes furent dans ce but préparées par la méthode d'Ogilvie ce qui permet de bien discerner les cellules A et B du tissu pancréatique.

Les résultats concernant l'ovaire sont exposés dans le tableau suivant:

Tableau N:o 2.

	Appareil folliculaire	Corps jaunes		Appareil folliculaire	Corps jaunes
N:o 1	+	++	N:o 11	+	+
2	+	+++	12	—	+++
3	+	+++	13	—	+++
4	—	+++	14	++	++
5	+	+	15	—	+++
6	++	+++	16	+	+++
7	+	+	17	+	+
8	++	+++	18	+	+
9	+	+	19	+++	+
10	+	+	20	+	+

La chose principale que saute aux yeux dans les ovaires est la lutéinisation excessive chez les animaux traités par l'alloxane. Cela se voit aussi bien dans les formes aiguës que dans les cas plus longuement observés, qui ont fait un diabète plus



Fig. 3.  
L'ovaire de la rate N:O 2.



ou moins grave. Dans les cas légers et les cas réfractaires il n'y a pas de dérangement notable entre les éléments folliculaires et lutéoides dans les ovaires, sauf chez une rate de contrôle qui présentait une hypertrophie folliculaire marquée. Il nous semble par conséquent que dans les cas, où le cycle ovarien est plus ou moins bloqué, cela se fait au stade lutéinique. Dans quelques ovaires l'appareil folliculaire fut indéniablement hypertrophié, mais dans les mêmes organes les corps jaunes furent comparablement encore plus développés et le rapport resta en faveur des éléments lutéiniques. Seulement dans un cas l'ovaire paraît être uniformément stimulée et dans trois cas relativement légers les ovaires n'ont pas présentées rien d'extraordinaire. — Chez trois animaux de contrôle le rapport folliculaire — lutéinique fut bien équilibré et l'aspect de ces organes fut complètement normal. L'hypertrophie folliculaire chez la quatrième rate de contrôle fut déjà mentionné auparavant.

En parcourant les préparations microscopiques du pancréas des animaux souffrant du diabète alloxanique il y a tout de suite une circonstance que attire l'attention sur elle et c'est la petitesse et la raréfaction des îlots de Langerhans. Quand on après les examine plus minutieusement à fort agrandissement on constate sans peine la diminution et parfois même la disparition des cellules B dans ces glandes. Souvent on voit aussi des cellules nécrotiques ou atteintes d'une dégénérescence hyalinique ou vacuolaire. Ces constatations sont d'ailleurs maintenant généralement connues et nous ne pouvons que les vérifier encore une fois. Le tissu pancréatique des rates de contrôle fut sans reproche.

## DISCUSSION

Premièrement nous avons pu constater en pratiquant la prise de sang analytique par ponction veineuse que cette manœuvre énervait quelquefois l'animal à un tel degré, que le taux du sucre sanguin montait notablement. Plus tard dans les ponctions ultérieures nous avons en général obtenu des valeurs normales. Ce fait a été entre autre signalé par *Vartiainen et al.* (1947).

Au cours de ce travail nous avons pu suivre l'effet d'un état diabétique sur le fonctionnement des ovaires chez la rate. Cette observation continue fut réalisable par les frottis vaginaux qui reflètent d'une façon très satisfaisante l'activité hormonale des glandes sexuelles féminines. Nous savons maintenant que le diabète alloxanique est d'origine pancréatique, un fait que nous avons personnellement pu constater par nos études histologiques. Il faut aussi noter que la réaction à l'alloxane diffère notablement d'un animal à l'autre, sans raison apparente.

L'effet principal du diabète alloxanique sur le cycle vaginal semble être le prolongement des périodes diestrales. Cela signifie un repos génital partiel. Le vagin des animaux a contenu toujours plus ou moins de mucus mélangé de polynucléaires pendant les phases diestrales. Cette trouvaille répond aux corps jaunes actives dans les ovaires. Nous avons pu confirmer ce dernier fait d'une manière convaincante par nos préparations histologiques sur les ovaires. La répercussion au niveau des glandes génitales ne s'installa pas immédiatement. Dans presque tous les cas une kératinisation vaginale se produisit encore pendant les trois premiers jours après le commencement de l'expérience, mais après cette courte période apparemment normale la kératinisation cessa de paraître pour plus ou moins longtemps. Cet-«amenorrhée» quasi initiale se produisit dans douze cas sur seize. Chez deux rates ce phénomène apparaîtra plus tard au cours du temps d'observation et deux animaux eurent jusqu'au bout un cycle régulier, malgré leur diabète.

La corrélation entre l'interruption du cycle vaginal et le taux du sucre sanguin montre que les rates peuvent avoir des kératinisations malgré une élévation remarquable de ce dernier. Le facteur nuisible pour l'activité ovarienne semble être la toxicose (acidose) diabétique. Dès que les animaux présentaient des signes toxiques, somnolence, lassitude, odeur acide, poil hérissé etc. les irrégularités cycliques commençaient. *R. Schröder* souligne déjà en 1930 l'influence des produits toxiques du métabolisme intermédiaire des diabétiques sur l'appareil génital. Encore, une investigation faite par un de nous, a démontré que chez l'homme les irrégularités menstruel-

les étaient beaucoup accentuées dans la catégorie de femmes faisant un diabète pancréatogène que dans les formes non-pancréatogènes. Ce fait semble à première vue surprenant parce que la grande majorité des syndromes diabétiques non-pancréatogènes est considérée comme d'origine hypophysaire et par conséquent l'on est enclin à admettre à la fois des troubles ovariennes causés par cette glande. Notre trouvaille inattendue peut cependant s'expliquer par le fait notoire que la tendance aux complications toxiques est infiniment plus accentuée dans la forme pancréatique que dans les formes non-pancréatiques du diabète sucré. Nos résultats obtenus chez le rat appuient cette hypothèse.

En plus nous voulons souligner que dans aucun cas nous n'avons pu noter une hyperactivité folliculaire à savoir kératinisation prolongée du vagin et encore moins du rut permanent. Donc il nous semble que le dérangement ovarienne dans ces conditions est toujours dirigé vers une prévalence lutéale.

Il faut aussi signaler que pendant le temps que nous avons suivi les animaux, jusqu'à huit semaines, nous n'avons pas pu constater une atrophie génitale ni macroscopique ni histologique. Il est néanmoins fort possible que le temps d'observation ait été trop court et qu'au cours d'un diabète chronique un état atrophique de l'appareil génital ait remplacé les conditions trouvées dans ce travail.

Le but du traitement insulinique au cours de cette expérience n'était pas d'établir des conditions normales mais seulement de prévenir une mort trop précoce et non souhaitable. Par conséquent nous ne pouvons pas nous exprimer sur le pouvoir de l'insuline de compenser l'effet alloxanique, mais dans plusieurs cas il nous paraît que la kératinisation se soit installée de nouveau sans doute comme résultat des piqûres insuliniques. L'expérience clinique humaine a aussi démontré, qu'un traitement insulinique adéquat est capable de soutenir un cycle menstruel régulier chez la femme diabétique.

## EN RESUMANT NOUS AVONS A NOTER:

L'effet du diabète alloxanique sur le cycle vaginal chez le rat est le prolongement des périodes diestrales jusqu'au blocage complet du cycle. Ce phénomène n'était pourtant pas constant et n'était pas en corrélation directe avec le taux du sucre sanguin. Une hyperlutéinisation ovarienne chez les rates diabétiques par contre était constante.

## SUMMARY

*K. E. U. Jäämeri and Helena Tarkiainen:*

*The vaginal cycle in alloxan diabetic rats.*

In rats suffering from alloxan diabetes a prolongation of the di-oestrous period and sometimes a complete cessation of the cycle was observed. The phenomenon was, however, not constant and no correlation with the blood sugar value seemed to exist. Hyperluteinization in the ovaries of the diabetic rats was regularly observed.

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## ON THE ARTIFICIAL INDUCTION OF OVIPOSITOR GROWTH IN THE BITTERLING (RHODEUS AMARUS BL.)

### I. SEASONAL VARIATIONS IN THE RESPONSE OF THE OVIPOSITOR TO PROGESTERONE

BY

B. DE GROOT and J. J. DUYVENÉ DE WIT<sup>1)</sup>

#### GENERAL INTRODUCTION

Outside the spawning season the female bitterling reacts to the administration of steroid hormones and a number of other substances by ovipositor growth (*Duyvené de Wit*, 1939; *Bretschneider & Duyvené de Wit*, 1941, 1947). As regards steroids, it has previously been shown that the effect on the ovipositor is indirect. The hormone is probably absorbed by the epithelium of the gills and acts (? via the hypothalamus) on the anterior hypophyseal lobe with the result that a luteogenic hormone is secreted which transforms eggs of a certain degree of maturity into »preovulation corpora lutea«. These corpora lutea produce the hormone (oviductin) which ultimately causes the ovipositor to grow.

The steroids, including progesterone, act via the hypophysis. However, oviductin and copulin (a hormone which,

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<sup>1)</sup> 31st Communication of the »Werkgemeenschap voor Endocrinologie«.

under certain conditions, is secreted into the water by male bitterlings) act on the ovipositor directly<sup>1</sup>).

The growth of the ovipositor, which occurs after the addition of active substances to the aquarium water, may readily be observed and measured with the naked eye, without necessarily entailing any disturbing systematic errors. Now the remarkable thing is that the average ovipositor growth of a number of sensitized and *selected* fishes (cf. p. 254), under certain constant external conditions has a *characteristic course*, when treated with certain steroids. The typical features in the ovipositor growth curves caused by pregnanes, androstanes, estranes and various allied substances, although subject to small fluctuations, yet maintain a strikingly peculiar character of their own. This is especially evident when comparing, on one and the same day, growth curves obtained with different substances.

Thus, Fig. 1 shows an arbitrarily selected pregnane-curve. The hours are marked along the abscissa, and the ovipositor growth, expressed in anal-fin units (A. U.), along the ordinate. One A. U. corresponds to  $\frac{1}{3}$  of the anterior radius of the spread anal-fin. In this growth curve the following characteristic features may be distinguished: (a) the time elapsing between the addition of the hormone to the water and the commencement of growth or »latent period«; (b) the period of linear growth following it, and (c) the total lengthening of the ovipositor at the end of the linear-growth period. Apart from these three features, the pre-history of the fish must be taken into account and this is reflected to some extent by (d) the

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<sup>1</sup>) It is thus doubtful whether oviductin and copulin possess a steroid structure. In our own view, the sexual hormones of fishes are quite different from those of mammals. This statement is perhaps equally applicable to the amphibia, and perhaps also to reptiles and birds. According to this view, the results obtained when these animals are treated with steroid mammalian hormones would, therefore, be of a pharmacological rather than of a physiological nature.

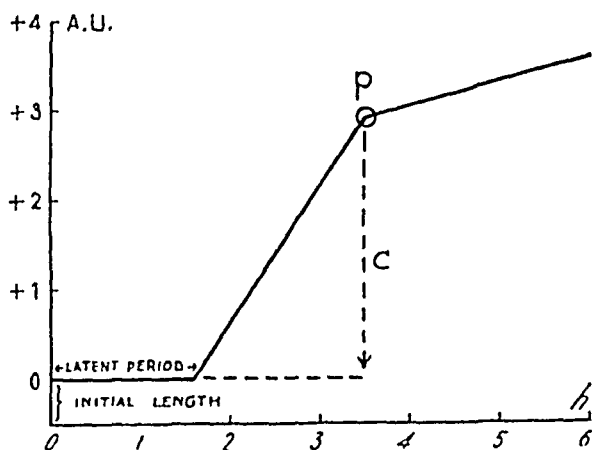


Fig. 1.

Ovipositor growth curve (theoretical).

c = quantitative growth.

p = terminal point of the period of initial growth.

starting length of the ovipositor at the commencement of each test.

The importance of this latter factor becomes evident when it is realised that the artificially induced ovipositor growth takes place at the expense of the stock of luteinizable eggs present in the ovary. The manner in which the ovipositor reacts to a given amount of hormone, therefore, is to a great extent dependent on the number of luteinizable eggs, and/or their degree of sensitivity to the luteogenic hormone. In fishes that have reacted more than about eight times in succession to active urine, this stock is gradually exhausted, with the result that the growth curves become increasingly flattened out, and lose their typical features. Also when fresh fishes are made to react for the first time, the typical features of ovipositor growth do not at once become manifest, since the ovary, in autumn and winter, must first be stirred from its condition of relative rest.

To bring the eggs to the degree of maturity in which they readily become luteinizable, a stimulus must be applied, i. e. sensitization. This is brought about by causing the fishes to react strongly, once or twice, to the active urine of pregnant



women. In such a urine a substance is present which has not hitherto been identified, but which has the remarkable property that it is hydrolyzed at room temperature in a few hours, a fact which is demonstrated by the reduction of the latent period from  $5\frac{1}{2}$  hours to about 2 hours<sup>1</sup>).

In the bitterling this luteidin causes a copious secretion of luteogenic hormone by the hypophysis, with the result that the eggs in the ovary acquire a certain degree of maturity. Thus a stock of 9—11 luteinizable eggs per section of the ovary is created, and this, it seems, is sufficient for the appearance of the typical features of the ovipositor-growth curves, features which may be obtained on a number of successive days with the aid of different steroids.

It has been shown experimentally that luteidin is eminently suitable for inducing this sensitization, because it is able — to a far greater extent than, e. g., progesterone — to maintain the contents of the ovary at a practically constant degree of sensitivity for one or more days.

To obtain growth curves with the most typical qualitative and quantitative characteristics, it is necessary, in addition to maintaining this constant degree of sensitivity, to select the right lengths of the ovipositors at the commencement of the experiment. For, as we shall see, the starting length has an influence on the duration of the latent period, on the duration of the linear growth, and on the quantitative result. It has been shown experimentally that a starting length of 3 A. U. gives the best results, but that fishes with ovipositors of 2, or 4 A. U. — providing they are present in equal numbers — may also be used successfully.

Bearing the foregoing in mind (i. e. selection as to starting

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<sup>1</sup>) This factor, which we (*D. d. W.*) have called *luteidin*, and which is probably a steroid, is excreted cyclically by non-pregnant women, the excretion rate rising during the corpus luteum phase to a level, similar to that found during pregnancy. The presence of luteidin has not been demonstrated in the urine of pregnant mares and cows; only rarely in the urine of the human male, and then only in low concentrations.

length and sensitization by luteidin), it is possible in fishes with a starting length of 2—4 A. U. to obtain ovipositor growth curves which, as will be shown again further on, are typical of the hormonal groups, and sometimes also of the kind of hormone which is added to the water. Naturally, only those hormones can be distinguished whose growth curves, or dose-responses are sufficiently different.

If, for example, we administer to different groups of fishes

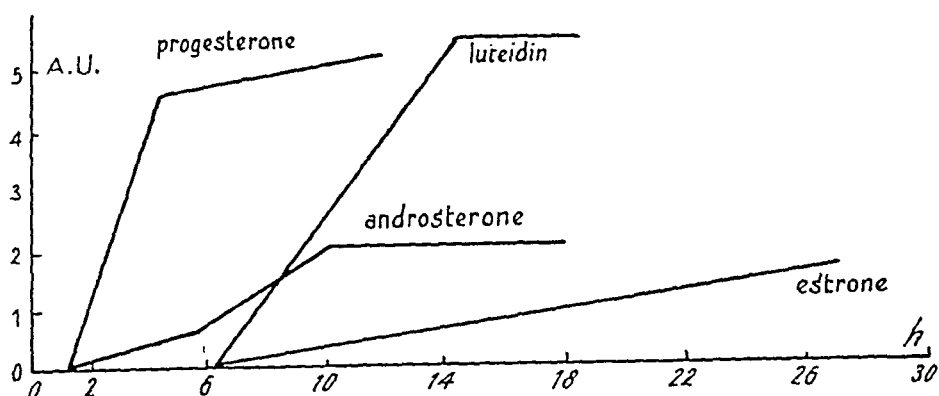


Fig. 2.

Ovipositor growth-curves obtained with relatively large quantities of progesterone, androsterone, luteidin, and oestrone. Note the typical differences in latent period and duration of linear growth.

progesterone, androsterone, oestrone and urine (luteidin), then we get the growth curves shown in Fig. 2, which do not derive their typical features (except, of course, the quantitative result) from the doses applied. It is striking that all pregnane derivatives tested up to the present, including the cortical steroids, show a maximum latent period of  $2\frac{1}{2}$  hours, while their maximum period of linear growth is 6 hours. In the case of androstanes the latent period is also short, but the time of linear growth is invariably longer, i. e. 8—12 hours. The latter substances, moreover, when administered in large doses, never produce the maximal ovipositor growth of the pregnanes. With most androgenic steroids there is a slight break in the curve at the  $5\frac{1}{2}$  hours-point, which is usually significant only with higher doses. The oestranes have a latent period of  $5\frac{1}{2}$

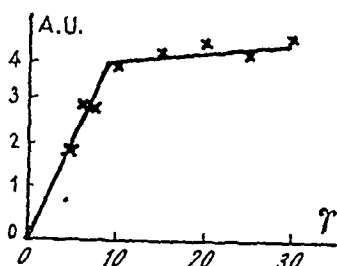


Fig. 3.

Progesterone; dose-response curve after  $4\frac{1}{2}$  hours ( $23^{\circ}$  C.).

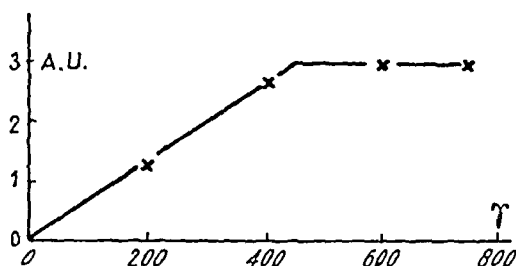


Fig. 4.

Corticosterone; dose-response curve after  $4\frac{1}{2}$  hours ( $23^{\circ}$  C.).

hours and their period of linear growth is extremely long, i. e. 42—54 hours. Non-hydrolyzed luteidin occupies a separate place owing to its latent period of  $5\frac{1}{2}$  hours and rapid growth within 6—7 hours.

For substances such as progesterone and corticosterone, whose growth curves differ only slightly, the dose response may sometimes show a distinct difference (Figs. 3 and 4). In view of the large number of known pregnane derivatives, the number of these substances showing a significant difference with regard to ovipositor growth, is naturally limited. This does not alter the fact that it is an extremely remarkable phenomenon that the bitterling should react in such a specific manner to pregnanes, androstanes, oestrans, and to a number of substances belonging to these groups, and that, by virtue of this, it might be used in certain suitable cases as an indicator of the presence of some special factor.

The object of our investigations is to endeavour to obtain a clearer insight into the processes involved in the artificial induction of ovipositor growth.

In the first place we thought it desirable to investigate the seasonal changes in the sensitivity of bitterlings not previously sensitized or selected.

In the second place the effects of different non-specific chemical agents and physical stimuli on the growth of the ovipositor were studied.

Finally, we endeavoured to discover the connection between the phenomena accompanying the artificial induction of ovipositor growth, and the adaptation syndrome (*Selye*, 1947), which should be regarded as an attempt on the part of the organism to offer resistance to the influence of non-specific harmful stimuli.

## SEASONAL VARIATIONS IN THE RESPONSE OF THE OVIPOSITOR TO PROGESTERONE

### MATERIAL AND METHODS

The bitterlings were caught in the autumn of 1947, and kept in a tank in the open air. At set times the number of fishes required for the experiment were taken out and brought to the laboratory, where they were placed in an aquarium at 17° C. For the actual experiment, they were then removed to small aquaria with 750 ml. water at 23° C. Each aquarium contained 3 fishes, which could be distinguished by their difference in size. In the morning 4  $\gamma$  progesterone dissolved in alcohol were added to each of 3 aquaria; 3 other aquaria, containing the solvent, served as controls. The duration of the test was 6 hours per day. After this the experimental and the control-fishes were separated individually and placed into water at 17° C., so as to be used again the next day. On December 16 and 19, 1947, January 6 and 27, February 13 and 27, March 8 and 23, and April 19 and 28, 1948 — i. e. 10 times in all — two new groups of 9 fishes were used each

time. Each experimental group was exposed, for 6—16 successive days for 6 hours per day, to the influence of progesterone. In no case was there any pre-treatment with luteidin (see general introduction).

## RESULTS

### (a) *Influence of the season on the length of the ovipositor.*

As Table 1 shows, the length of the ovipositors, before the commencement of the 10 successive tests, increases from the

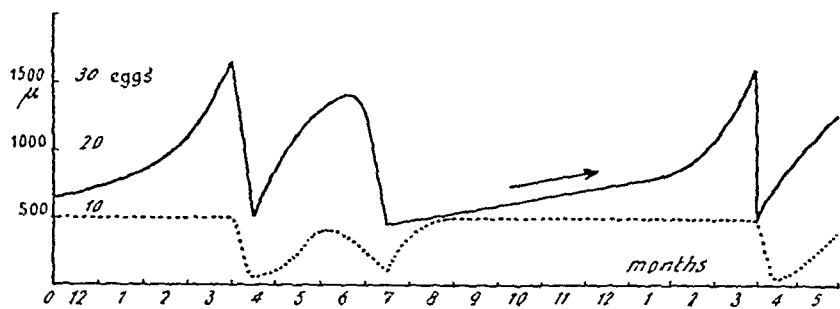


Fig. 5.

———— = diameter of large ova.

..... = number of large ova.

The relation between the different months of the year (abscissa) and the average number of large ova per section, with their diameter (ordinate). The diameter of the large ova increases only slowly during the autumn and winter months (——).

autumn to the spring, i. e. from 0.3 to 8.9 A. U. (see also Meltzer, 1947). The gradually increasing production of oviductin, which is probably accountable for this, may be the result of two factors:

- (1) increasing sensitivity of the large eggs to the luteinization hormone, or
- (2) an increase in the number of large luteinizable eggs.

It has been shown by the investigations of Bretschneider & Duyvené de Wit (1947) that it is the first of these two factors that must be held responsible for the increased oviductin production. The increasing sensitivity is coupled with a gradually increasing size of these ova, whose diameter increases from 600 to 850  $\mu$ .

Fig. 5 clarifies this statement; along the abscissa the months of the year are indicated, and along the ordinate the number of large eggs per section, and their diameter.

It will be seen that the number of large eggs per section

Table 1.

Date	Average length of ovipositors of 18 fishes, in A. U.
16 Dec. 1947	0.3
19   "   "	0.7
6 Jan. 1948	0.6
27   "   "	0.6
13 Feb.   "	2.3
27   "   "	0.9
8 Mar.   "	4.3
23   "   "	5.3
19 Apr.   "	3.3
28   "   "	8.9

remains constant from August to April; but that the diameter of these ova gradually increases *in direct proportion to the lengthening of the ovipositor* (Table 1).

Not only does the starting length on the first day of each test change as the season advances, but the starting length in the first 3—5 testing days of each series also changes; it increases progressively. At the end of December, January and February these increases amounted, respectively, to 1.8, 3.0 and 5.0 A. U. The ovipositor growth during the first 3—5 testing days appears to become greater for each series as the season advances. This difference is probably the result of an increasing sensitivity of the ova to the gonadotrophic pituitary factor. The reactions on the first testing day of the respective series, on the contrary, do not show any distinct differences.

The above phenomena were observed not only with progesterone but also with pregnenolone and boiled urine (hydrolyzed luteidin).

(b) *The influence of daily repeated administration of progesterone on the growth of the ovipositor.*

The ovipositor curves of fishes that have been exposed on a number of consecutive days (for 6 hours per 24) to 4  $\gamma$

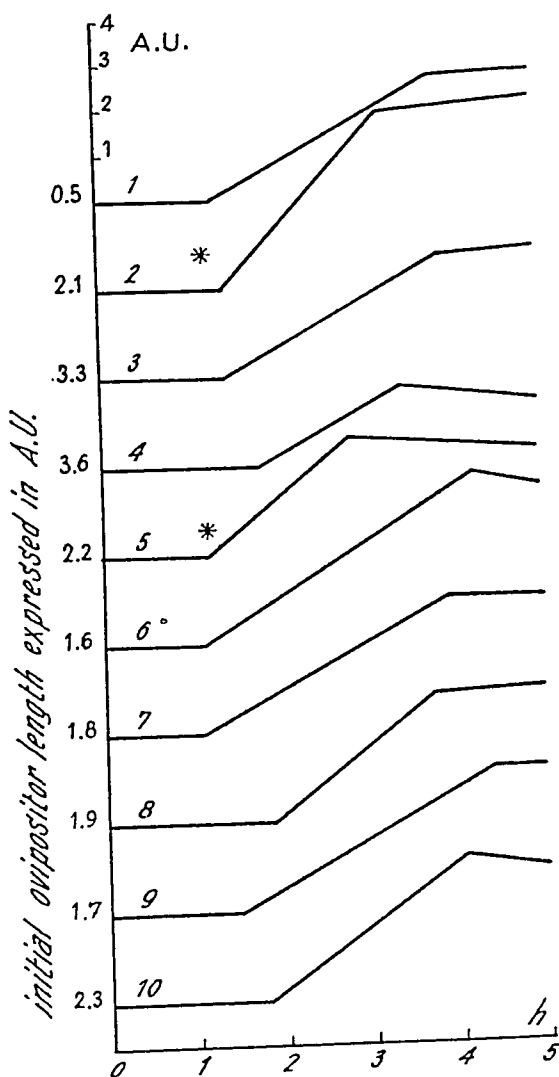


Fig. 6.

Ten growth-curves obtained by the action, on 10 successive days and during 6 hours out of 24, of 4  $\gamma$  progesterone, on non-sensitized and unselected bitterlings (23° C.). The variable starting lengths of the ovipositors are expressed in figures to the left of the ordinate.

progesterone show certain differences within one and the same series, which, in the 10 series examined by us, repeat themselves with some regularity. As these differences can plainly be seen in the series started on January 27, 1948, we shall confine ourselves to reproducing the growth curves found in this experiment (Fig. 6).

The features which deserve special attention (see Fig. 1) are:

- (1) the duration of the latent period;
- (2) the moment at which the linear growth stops;
- (3) the ovipositor growth as measured at the end of the period of linear growth, and
- (4) the length of the ovipositor at the start of each test.

It will be seen from the graph that the latent period shows fluctuations during the course of the 10 successive testing days. (We may mention in passing that such fluctuations were never observed in fishes that had previously been sensitized with luteidin.) These fluctuations occur within a period of from 1 to 2 hours. The number of hours of the latent period, plus the period of linear growth, varies from 3 to 4½ hours. (It must be noted in this connection that the curves marked with an asterisk fall somewhat outside the picture, owing to their too steep course, as will be shown later.) Again it appears that the total lengthening at the end of the linear growth period reaches different values on different testing days. Finally, the average starting length on the successive days also varies.

The question naturally arises as to whether there exist correlations between these four different values. To this question Fig. 7 provides an answer. In this graph, along the abscissa, the 10 testing days are marked on which the curves in Fig. 6 were obtained. Along the ordinate are marked: (I) the latent period in hours, (II) the terminal point of the linear growth period in hours, (III) the increase in growth during the period of linear growth, in A. U., and (IV) the increase in the initial length on the respective testing days, in A. U.

If we take the latent period (I) as a basis, then it appears



that the terminal point of linear growth (II) comes later when the latent period is shorter, and vice versa. This correlation is not expressed in the two curves marked with an asterisk. For some unknown reason, these two curves, of the second and fifth testing days respectively, fall outside the general interrelation.

If we compare the latent period (I) with the number of

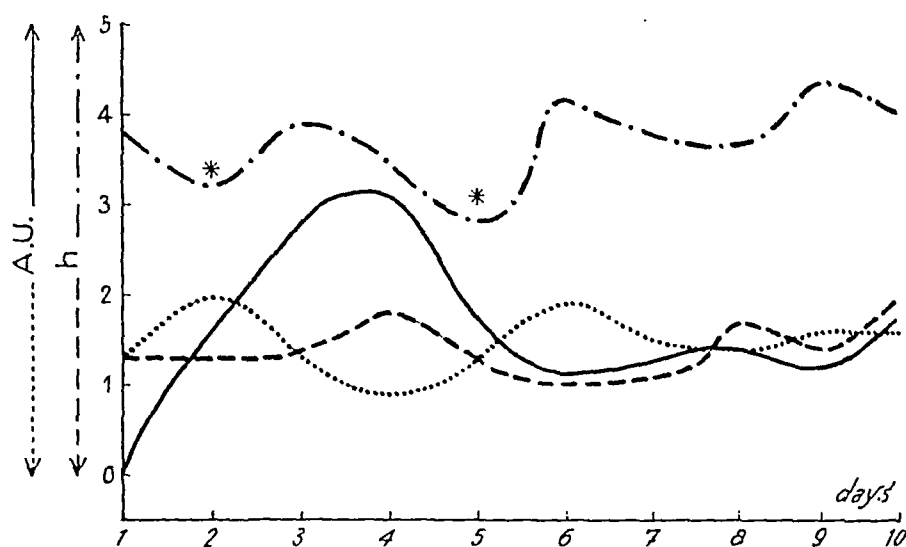


Fig. 7.

Series of Jan. 27, 1948.

- = latent period in hours (I);
- . - . - . = terminal point of the linear growth period in hours (II);
- ..... = quantitative growth in A.U. (III);
- = increase in initial length in A.U. (IV).

These four values are plotted against the time (abscissa), for each curve from Fig. 6.

growth-units at the end of the period of linear growth (III), we shall again note a distinct correlation, resembling, this time, a mirrored reflexion: as the latent period becomes longer, the ovipositor growth caused by a given quantity of progesterone becomes smaller. Although these differences in

growth remain within the range of only 1 A. U., calculation nevertheless shows them to be significant.

The increase in the starting length (IV) runs parallel with the duration of the latent period (I).

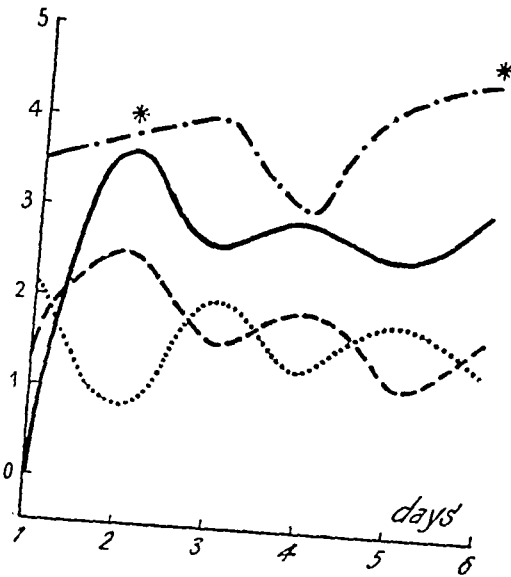


Fig. 8.

Series of Dec. 19, 1947. Explanation as in Fig. 7.

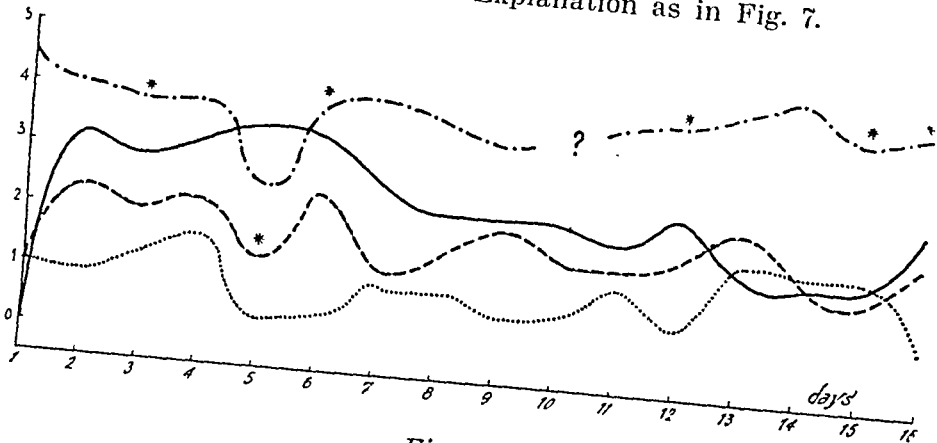


Fig. 9.

Series of Feb. 27, 1948. Explanation as in Fig. 7.

The series started on December 19, 1947, shows the relations observed in Fig. 7 most clearly (Fig. 8), and the series of February 27, 1948, least clearly (Fig. 9). The series of January 27, 1948 (Fig. 7) therefore shows an average picture.

The deviations in some curves marked with an asterisk are caused by the fact that the periods of first and second growth show a very obtuse angle and this increases the spread of the points of observation.

If the fishes are not transferred every day from  $17^{\circ}$  to  $23^{\circ}$  and from  $23^{\circ}$  to  $17^{\circ}$ , but kept constantly at  $23^{\circ}$  in order to eliminate the heat effect (see next paper), then the rhythmic, interdependent change in the magnitudes mentioned will also appear here (see Fig. 10). Owing to the constantly higher temperature the fishes are evidently activated more intensely, with the result that the exhaustion stage is reached sooner.

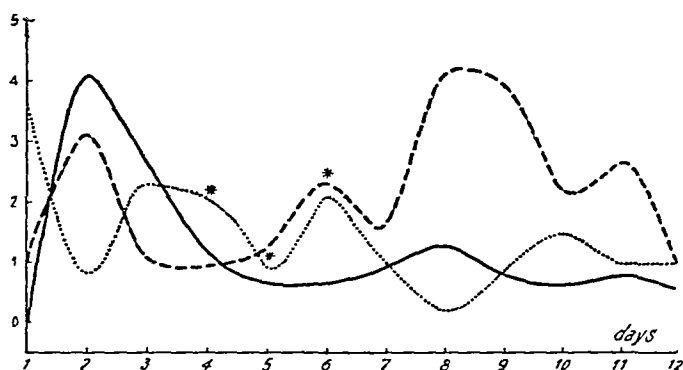


Fig. 10.

Series without heat effect (see text). Explanation as in Fig. 7.

## SUMMARY

1. From autumn until spring it appears that the initial length of the ovipositors of female bitterlings, caught in their natural state, gradually increases.

2. As the season advances, the starting length of fishes treated daily with progesterone increases progressively during the first 3—5 testing days.

3. The ovipositor growth was expressed as growth curves which were obtained from non-sensitized and non-selected bitterlings that had been exposed to progesterone on 6—16 successive days, for 6 hours daily. According to the order of sequence of the testing days, the curves show certain differences, in which the following relations were found: as the ovipositor

at the start of the test becomes longer, the latent period of the growth curve increases, the linear growth period is shorter and the growth of the ovipositor less.

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## ON THE ARTIFICIAL INDUCTION OF OVIPOSITOR GROWTH IN THE BITTERLING (RHODEUS AMARUS BL.)

### II. OVIPOSITOR GROWTH CAUSED BY DIFFERENT CHEMICAL AND PHYSICAL AGENTS

BY

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#### INTRODUCTION

As already mentioned in the preceding publication (*de Groot & Duyvené de Wit*, 1949: general introduction), the female bitterling reacts to the steroids of the pregnane, oestrane and androstane groups. Although the fishes are very sensitive to these substances, and especially to the pregnanes, they also react to various other substances of quite a different chemical constitution, although the active doses of the latter is generally higher than that of the former, while the growth curves they produce are also of a somewhat different type from those of the pregnanes.

As early as 1939, one of us (*D. d. W.*) was struck by the fact that both progesterone and testosterone, when administered in excessive doses, act injuriously, i. e. produce symptoms of

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narcosis. With progesterone these phenomena occurred after much smaller doses than with testosterone, and progesterone was also far more active in the ovipositor test than testosterone. This fact was the more interesting in view of *Selye's* observation that in the rat progesterone also has a very powerful anesthetic action as compared with other steroids. This raised the question as to whether the capacity of different substances and physical stimuli to induce ovipositor growth is based on their (unspecific) harmfulness, or (in the restricted sense) on their narcotic action.

To settle this point it was necessary to test different chemical substances and physical stimuli for their capacity to produce ovipositor growth.

### EXPERIMENTS

The different degrees of anesthesia to be distinguished in fishes were determined empirically, and given an index figure (0—5):

- 0 = no anesthesia, or at most a slight folding together of the fins or darker colour;
- 1 = complete discolouration, excitation, and a beginning of passivity;
- 2 = the fish sways with the movement of the water, with an occasional spontaneous movement;
- 3 = beginning of lateral position;
- 4 = complete anesthesia in lateral position, with good respiration;
- 5 = completely lateral position, irregular respiration, possibly ending lethally.

Although these distinctions are of a more or less subjective character, and although the different stages cannot be observed or separated equally clearly, this classification proved sufficiently accurate in practice to allow of a comparison to be drawn between the depth of the narcosis and the growth of the ovipositor.

The experiments were made with sensitized fishes; by selection, the starting length of their ovipositors was 2—4

A. U. A separate group of bitterlings was treated with 8  $\gamma$  progesterone per 750 ml. water (23° C.), as control for the sensitivity of the fishes. When volatile substances such as ether were used, the aquaria were covered as carefully as possible with a glass plate.

In the following diagrams the course of the narcosis is shown by a broken line, and that of ovipositor growth by an unbroken line. The figures indicate the degree of narcosis.

(a) *Chemical stimuli.*

Previous investigations (*Bretschneider & Duyvené de Wit*, 1947) had already shown that the following substances were inactive: yohimbine, digoxigenin, vitamin D<sub>2</sub>, galactose, maltose, saccharose, glycogen, gelatin, casein, glycine, ascorbic acid, glucose, and  $\alpha$ -tocopherol acetate. The following were active: ethanol, butanol, veratryl alcohol, glycerol, glycocholic acid, desoxycholic acid, sodium taurocholate and taurine.

Other substances tested:

(i) *Metal ions: Mg<sup>++</sup>.* Slow infusion of MgSO<sub>4</sub> in mammals first causes paralysis of the terminals of the motor nerve fibres. For the bitterlings we used a 2½ per cent solution. This resulted in a slight narcosis (grade 1). After 12 hours, the ovipositors of the experimental animals had been reduced in length, on the average to 0.5 A. U. less than those of the control-fishes, possibly indicating slight »growth«.

(ii) *Bromine compounds: NaBr.* This acts in mammals as a hypnotic, and, in higher doses, also as an anesthetic. The sub-lethal dose could not be accurately determined in the case of the bitterlings, as the lateral position on the bottom of the tank did not set in until after 16 hours. The anesthesia was irreversible, and after administration of a 2 per cent solution the fishes eventually died. NaBr, in the concentration mentioned, produced a very protracted ovipositor growth with a short latent period (see Fig. 1).

(iii) *Other inorganic compounds: nitrous oxide.* A volatile narcotic with an initially exciting action. The gas was administered through a flask filled with water at 23° C. until a

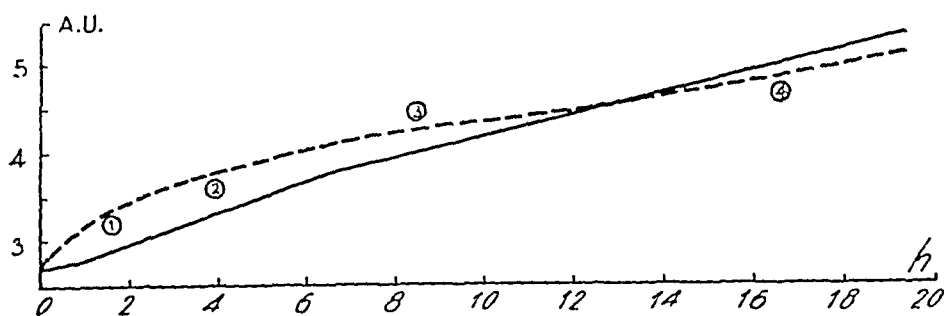


Fig. 1.

Anesthesia and ovipositor growth after administration of sodium bromide.

saturated solution was obtained. The bitterlings were then placed into this solution. Air was pumped slowly through the water. Narcosis (grades 1—2) set in; after 20 hours only very slight ovipositor growth could be observed (1 A. U.).

(iv) *Hydrated carbon compounds: cyclopropane.* A very volatile narcotic, readily soluble in water. The cyclopropane was administered through a glass bottle or flask filled with water at 23° C. After the fishes had been added, ample quantities of oxygen were immediately pumped through. Narcosis set in very rapidly (grade 5); recovery, too, took place again quickly. The narcotized fishes were found both on the bottom and at the surface. Only by slowing down their recovery from narcosis was it possible to obtain ovipositor growth in a number of fishes (see Fig. 2).

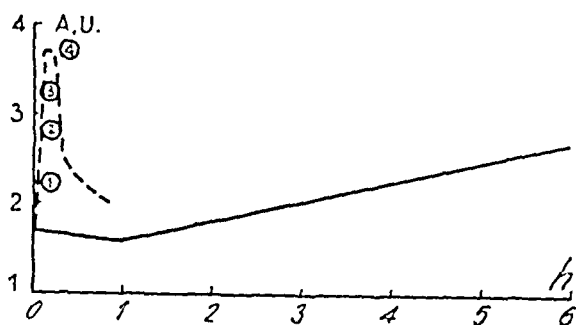


Fig. 2.

Anesthesia and ovipositor growth after administration of cyclopropane.



(v) *Camphor*. This substance acts, in mammals, by stimulating the central nervous system, causing convulsions. Administration in large doses causes paralysis. Administration in a 0.007 per cent concentration caused the fishes to become dark in colour, and to adopt the lateral position. There was also an increase in the size of the ovipositor (see Fig. 3).

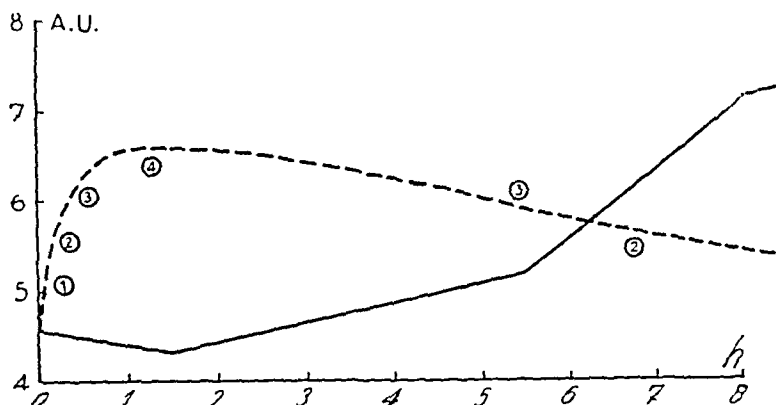


Fig. 3.

Anesthesia and ovipositor growth after administration of camphor.

(vi) *Chlorine compounds: chloroform*. Is often used as a narcotic together with ether, and, like ether, produces in mammals a marked narcosis passing from the central to the peripheral parts of the nervous system. Administration in a 0.015 per cent concentration produced a marked growth of the ovipositor (see Fig. 4).

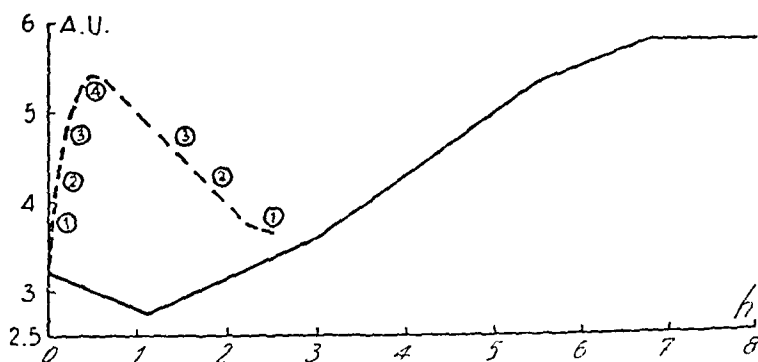


Fig. 4.

Anesthesia and ovipositor growth after administration of chloroform.

(vii) *Alcohols: ethanol*. An anesthetic which, in a 1.3 per cent solution, also acted as a violent excitant on bitterlings, producing ovipositor growth (see Fig. 5).

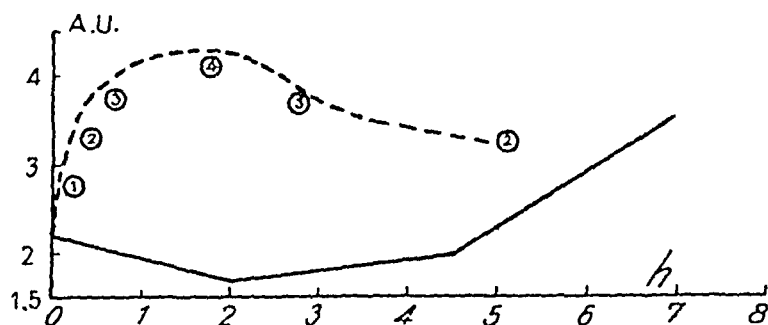


Fig. 5.

Anesthesia and ovipositor growth after administration of ethanol.

(viii) *Avertin*. A basal anesthetic with a strongly blocking action on the synapses. A 0.01 per cent solution produced narcosis of long duration, with lateral position of the fishes on the bottom of the tank, and ovipositor growth (see Fig. 6).

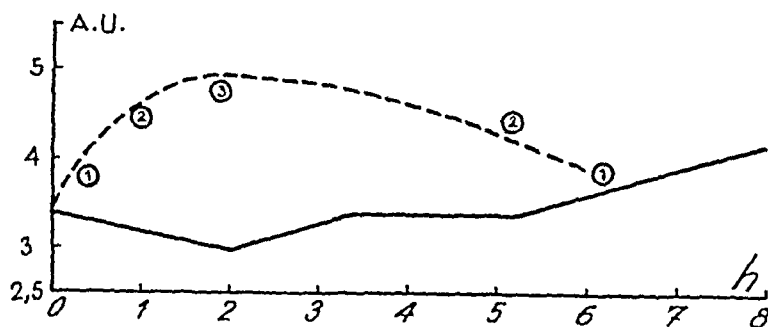


Fig. 6.

Anesthesia and ovipositor growth after administration of avertin.

(ix) *Aldehydes: paraldehyde*. A narcotic, not readily soluble in water. With 0.05 per cent, no lateral position was obtained. The narcosis picture was indistinct; there was ovipositor growth (see Fig. 7).

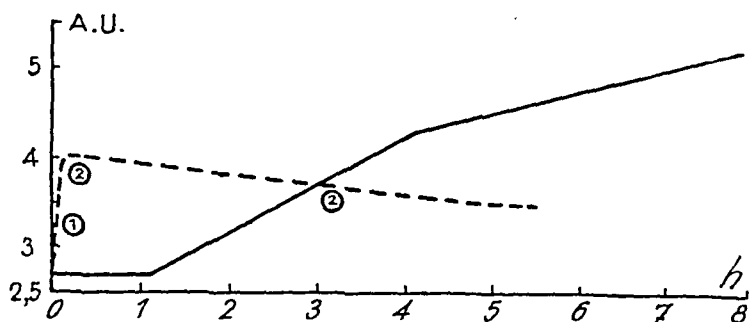


Fig. 7.

Anesthesia and ovipositor growth after administration of paraldehyde.

x) *Ketones: acetone*. Has narcotic properties. In a 0.9 per cent concentration it produced both anesthesia and ovipositor growth (see Fig. 8).

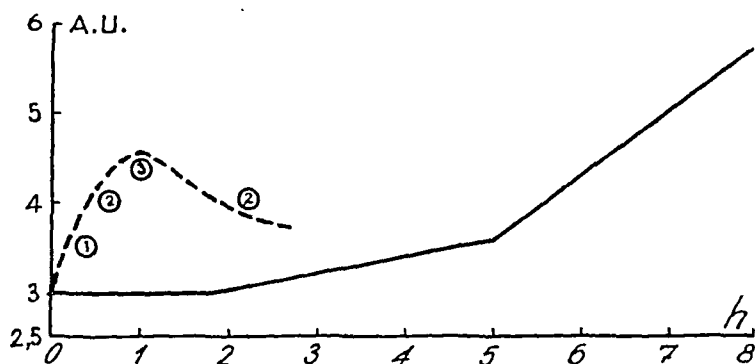


Fig. 8.

Anesthesia and ovipositor growth after administration of acetone.

(xi) *Ethers: dimethylether*. Anesthesia and ovipositor growth were obtained with ether in an initial concentration of 0.5 per cent (see Fig. 9).

(xii) *Urethanes: ethylurethane*. An anesthetic with an irregular action. With 0.1 per cent, ovipositor growth was obtained but no lateral position (see Fig. 10).

(xiii) *Barbituric acid derivatives: evipan*. A very rapidly acting anesthetic. This substance had to be injected intraperitoneally in the bitterlings. Narcosis is complete within a few

minutes, soon followed by recovery. 0.25 mg. per fish produces ovipositor growth (see Fig. 11).

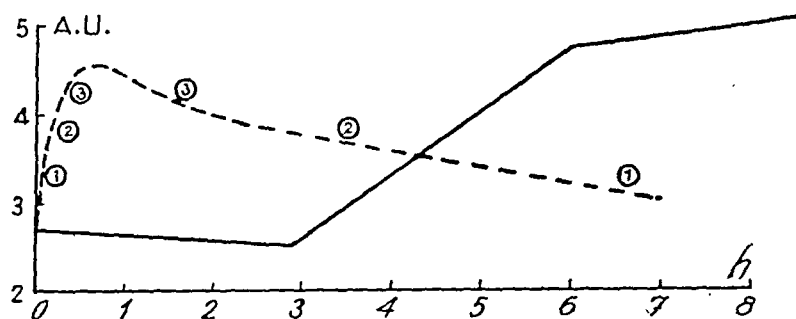


Fig. 9.

Anesthesia and ovipositor growth after administration of dimethylether.



Fig. 10.

Ovipositor growth after administration of ethylurethane. The grades of narcosis could not be estimated accurately.

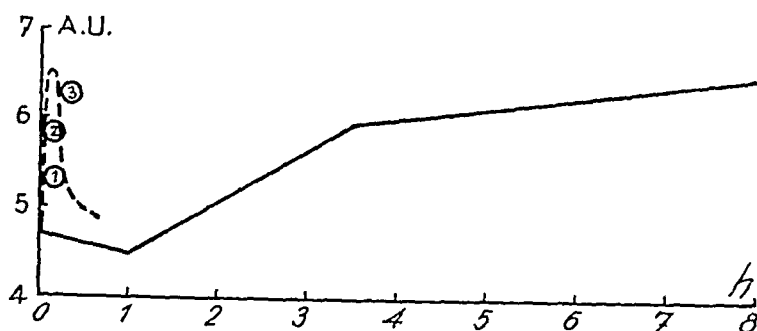


Fig. 11.

Anesthesia and ovipositor growth after administration of evipan.

*Luminal*. A hypnotic with a weak narcotic action. In a 0.04 per cent solution it causes distinct narcosis in bitterlings only after 16 hours, and without the lateral position. For ovipositor growth see Fig. 12.

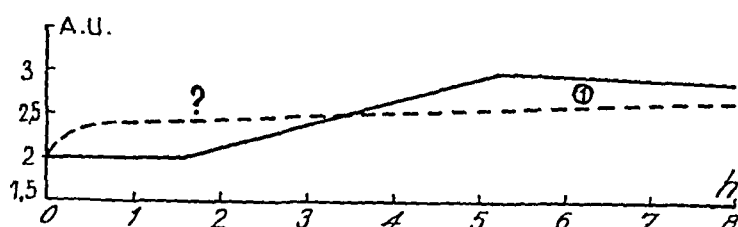


Fig. 12.

Anesthesia and ovipositor growth after administration of luminal.

*Veronal*. A hypnotic; dissolved in a little alcohol, and added to water to form a 0.001 per cent solution, it causes a narcosis whose course is difficult to determine. The fishes die after a few days because of cumulative action; for ovipositor growth see Fig. 13.

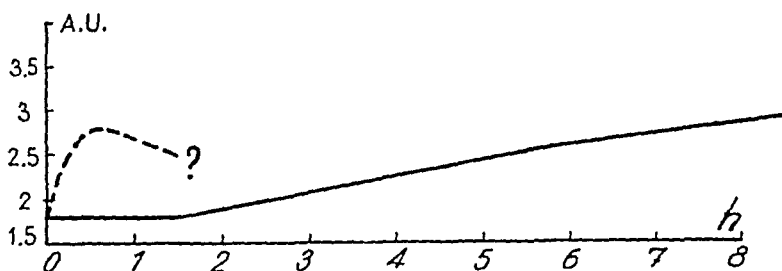


Fig. 13.

Anesthesia and ovipositor growth after administration of veronal.

(xiv) *Benzene derivatives: novocaine*. A local anesthetic whose secondary effects are less injurious than those of cocaine. A 0.005 per cent solution produces ovipositor growth (see Fig. 14).

(xv) *Pyramidon*. Dimethylaminopyrine is an antipyretic which in small doses causes paralysis, and in high doses, excitation of the medullary centres. It does not produce narcosis

of the central nervous system, but it augments the narcotic effect of different hypnotics. When administered to bitterlings in a 0.03 per cent concentration it produces ovipositor growth (see Fig. 15).

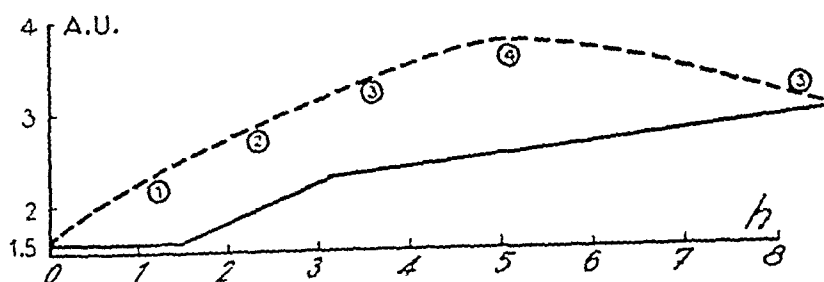


Fig. 14.

Anesthesia and ovipositor growth after administration of novocaine.

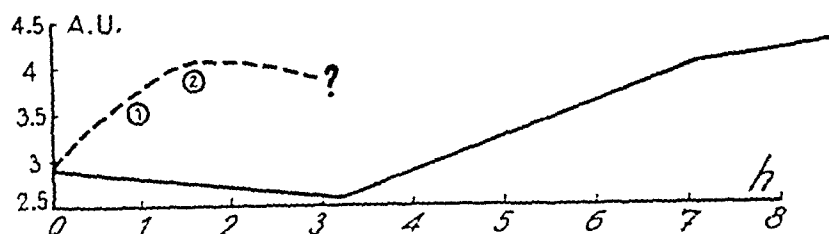


Fig. 15.

Anesthesia and ovipositor growth after administration of pyramidon.

(xvi) *Antifebrins: acetanilid*. An antipyretic with local anesthetic action. In a 0.02 per cent concentration it produces, only in fishes with ovipositors of a high starting length (minimum 5 A. U.), a lengthening by 3 A. U. in 8 hours.

(xvii) *Pyridine derivatives: coramine*. Has the same action as camphor. It also acts as an excitant on the spinal cord. A 0.007 per cent concentration produced a darker colouring of the fishes, but no lateral position. For ovipositor growth see Fig. 16.

(xviii) *Alkaloids: cocaine*. A local anesthetic, more especially for the peripheral nerves. Its action on the central nervous system is injurious to the centres of the lumbosacral cord.

A 0.003 per cent concentration produced lateral position on the bottom of the tank and ovipositor growth (see Fig. 17). In contrast to the other substances tested, there was contraction of the chromatophores, which caused the fishes to become extremely pale in colour. Spasmodic symptoms also occurred.

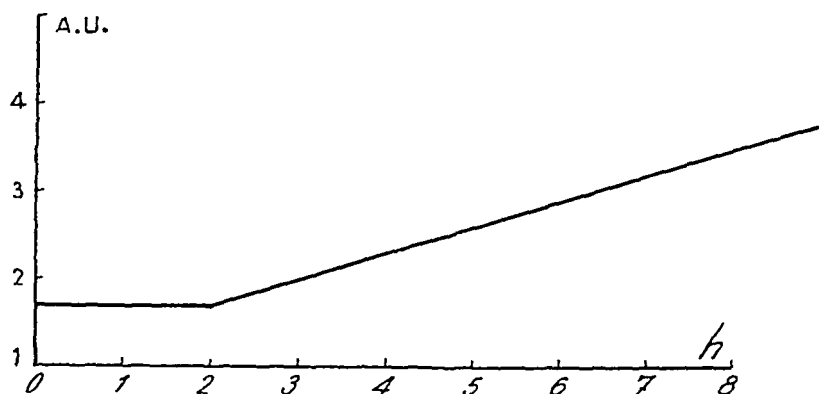


Fig. 16.

Anesthesia and ovipositor growth after administration of coramine.

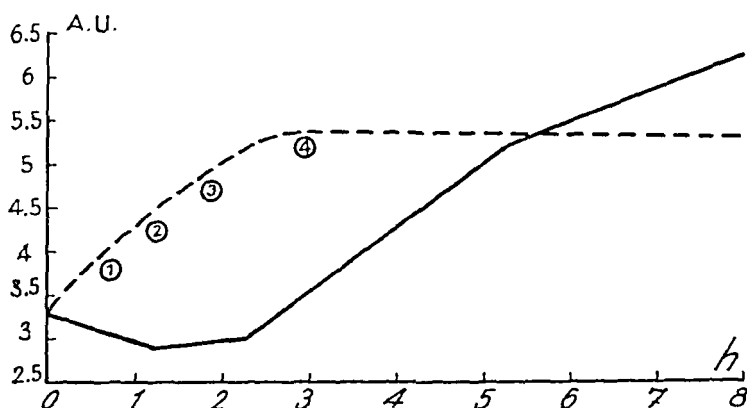


Fig. 17.

Anesthesia and ovipositor growth after administration of cocaine.

(xix) *Phenanthrene derivatives: morphine*. Produces different reactions in various species of animals. Either stimulation or paralysis of the central nervous system may occur. Injection of 0.3—0.7  $\gamma$  per fish produced a dark colour, lateral position, and ovipositor growth (see Fig. 18).

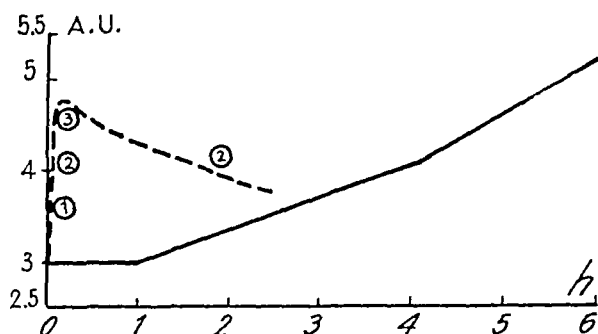


Fig. 18.

Anesthesia and ovipositor growth after administration of morphine.

(xx) *Steroids: progesterone*. When injected in relatively high doses, steroids act anesthetically in mammals (*Selye*); in small doses they produce different specific pharmacological effects. A 0.00004 per cent progesterone solution causes, in bitterlings, lateral position on the tank-bottom and ovipositor growth (see Fig. 19). Progesterone and desoxycorticosterone acetate are, in the bitterling, the most active anesthetics we have so far been able to find.

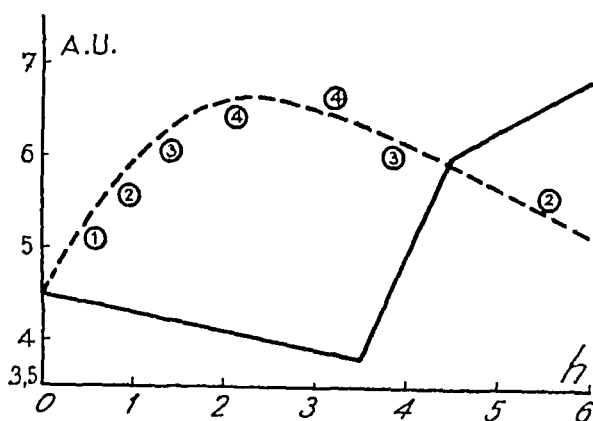


Fig. 19.

Anesthesia and ovipositor growth after administration of progesterone.

(xxi) *Non-specific toxic substances: formalin, mercuric chloride, antistin and picric acid*. When administered in sub-lethal doses, neither anesthesia nor ovipositor growth was obtained.



(b) *Physical stimuli.*

(i) *Heat.* When bitterlings caught in the natural state are placed in water at  $23^{\circ}\text{C}$ ., so that they are subjected to a temperature-shock of  $10^{\circ}$ , alarm phenomena occur which are manifested in violent movements of the gills, disturbances in the equilibrium, a dark colour and shortness of breath. In non-sensitized bitterlings the temperature-shock is soon followed by protracted ovipositor growth, which has been described by one of us (*D. d. W.*) as »autonomous« (see Fig. 20). In sensitized fishes, the heat-shock at first produces a slight shortening of the ovipositor and this is followed by only slight growth (see Fig. 21).

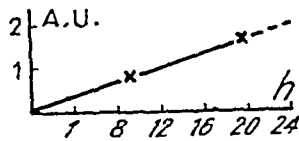


Fig. 20.

Autonomous ovipositor growth caused by heat shock ( $3-23^{\circ}\text{C}$ .) in non-sensitized fishes.

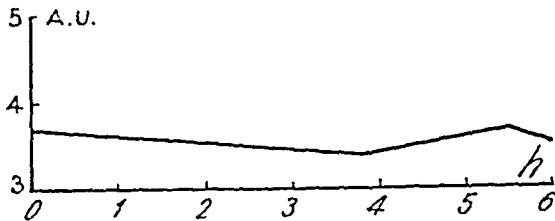


Fig. 21.

Autonomous ovipositor growth caused by heat shock in sensitized bitterlings.

(ii) *Cold.* A lowering of the temperature from  $23^{\circ}$  to  $3^{\circ}\text{C}$  is tolerated, provided the fishes are helped to survive the shortness of breath by means of a dose of coramine which, in itself, is inactive. After  $\frac{1}{2}$  hour the fishes have recovered from the shock. No change in the length of the ovipositor is observed within 12 hours after this.

(iii) *Trauma.* When the bitterlings are wounded, or dropped, the males very soon show the »nuptial« colours, and the

females a dark colour as the manifestation of an alarm reaction. In the females no change in the ovipositor length was observed during the subsequent observation period.

(iv) *Light*. In previous experiments one of us (*D. d. W.*) had already noticed that, under the influence of strong illumination, the reaction to certain quantities of progesterone is increased by 20—25 per cent, without change in the characteristics of the growth curves typical of this steroid. This effect has now been confirmed. Further, when sensitized fishes were subjected to strong light alone, ovipositor growth occurred after a latent period of  $5\frac{1}{2}$  hours (see Fig. 22).

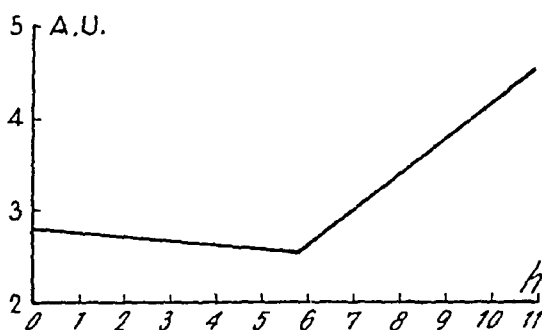


Fig. 22.

Ovipositor growth in sensitized fishes caused by continual strong illumination.

## DISCUSSION

It appears from the above experiments that some more or less harmful substances and stimuli do produce ovipositor growth while others do not. Ovipositor growth, therefore, is not a *necessary* consequence of the action of an injurious stimulus as has erroneously been postulated by *Van Koersveld* (1947). It appears that the causative agents producing ovipositor growth are limited more especially to steroids, non-steroid anesthetics, heat, and light.

At the present stage of our investigations, therefore, we can distinguish the following groups of chemical and physical stimuli:

- (I) Substances and stimuli producing no narcosis and no ovipositor growth:
  - (a) innocuous agents, e. g. glycogen;
  - (b) injurious agents, e. g. mercuric chloride, antistin (Ciba), cold-shock, and trauma.
- (II) Substances and stimuli producing narcosis but no ovipositor growth have not, so far, been found.
- (III) Substances producing both narcosis and ovipositor growth, e. g. ether, and steroids in relatively large doses.
- (IV) Substances and stimuli producing ovipositor growth but no clearly observable narcosis, e. g. some non-steroid anesthetics, steroids in small doses, heat-shock, and strong light.

The ovipositor growth occurring under natural conditions during the spawning season is caused by the corpus luteum hormone of the fish (oviductin<sup>1</sup>). In common with the copulin<sup>1</sup>) secreted by the male bitterling, oviductin acts directly on the ovipositor, and there is no reason whatever to assume that these substances, in physiological doses, are felt by the animals to be in any way harmful. We might therefore class both oviductin and copulin with group IV.

It is striking that the steroid and the non-steroid narcotics occur both in group II and in group IV. This implies that both groups comprise substances able to cause ovipositor growth when administered either in narcotic or in sub-narcotic doses. Nevertheless there is a difference, in that the steroid doses which only just induce ovipositor growth (and this applies especially to the pregnancies) are many times smaller (e. g., progesterone, 75 times smaller) than those which only just produce narcosis; whereas the narcotic doses of the non-steroid anesthetics tested by us, may be estimated to be, at most, 10

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<sup>1</sup>) See the general introduction in the preceding paper (*de Groot & Duyvené de Wit*, 1949).

times higher than those which only just induce ovipositor growth.

The following table shows the approximate ratios between the doses which only just produce narcosis and those which only just produce ovipositor growth, respectively, by means of an index figure, in which the steroids and non-steroids are classed as follows:

index narcosis: ovipositor growth	substances
1: 1—10	non-steroid narcotics
1: 20	steroids (androstanes)
1: 75	steroids (pregnanes)

It is therefore evident that the relative point in the dosage at which ovipositor growth appears without observable narcosis, is spread over a much wider range for the steroid than for the non-steroid narcotics.

We shall now consider what causes the difference in the action of steroids at the higher and the lower doses.

As is seen from our experiments, the latent period is longer in the case of steroids (for instance, progesterone) when administered in large doses, as the narcosis lasts longer. In the case of steroids in smaller dosage, however, the situation is quite different. Different quantities of, e. g., progesterone, produce, within the sub-narcotic range, and when tested on one and the same day, quantitatively different growth curves, with short and equal latent periods. Prolongation of the latent period appears only when, by excessive dosing, the narcotic limit is exceeded, and also when, simultaneously with a small quantity of progesterone, a narcotically-active quantity of, for instance, avertin is added (see Fig. 23). These differences might be explained by assuming that steroids in larger doses have, to some extent, other points of attack in the bitterling's organism than steroids in smaller doses.

We are inclined to think that, under the influence of a ste-

roid in small, sub-narcotic dosage, only one — or a combination of a few — centres («the sexual centre») of the central nervous system is blocked; so that the hypophysis (or, possibly, the hypothalamus, which is superimposed on it) is uninhibited and starts acting independently, with the result that gonadotrophic hormone is secreted, together with increasing oviductin production and ovipositor growth.

In increased, narcotizing doses, however, both the hypophysis and the entire central nervous system are blocked,

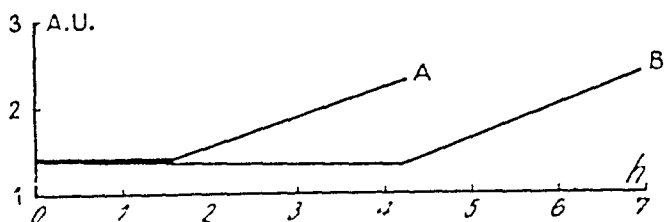


Fig. 23.

A = growth curve after administration of progesterone.

B = " " " " " " " plus a narcotizing quantity of avertin.

which, on the one hand, is manifested by an interruption of the production of gonadotrophin, and on the other, by narcotic symptoms. Thus, before complete narcosis appears and after it is ended, there is a stage in which the blocking is restricted to the «sexual centre» which regulates the hypophysis, and in which ovipositor growth can set in.

Generally speaking, there is this essential similarity between the non-steroid narcotics and the steroids, that both of them, in sub-narcotic doses, block the sexual centre and may, therefore, produce ovipositor growth without narcosis, while, in large doses, they also block the hypophysis and the entire central nervous system, causing narcosis without ovipositor growth.

There is, however, this remarkable difference between the non-steroid narcotics and the steroids — theoretically speaking — that the affinity of the sexual centre for steroids is considerably greater than that for the non-steroid narcotics,

which, in their turn, have a relatively greater attraction for the other tissues of the central nervous system. The selective affinity of the sexual centre for steroids (and especially pregnanes) might therefore explain the fact that these substances produce ovipositor growth in doses far below those which produce the narcotic effect, whereas the non-steroid narcotics, whose affinity for the sexual centre and for the other parts of the central nervous system differ only slightly, can cause ovipositor growth only when administered in a dose equal, or nearly equal to the narcotic dose.

This »disinhibition hypothesis« was supported by the following observations.

(1) With substances such as NaBr (Fig. 1) and novocaine (Fig. 14), with slowly increasing narcosis, ovipositor growth soon sets in, and its intensity decreases as narcosis becomes more complete. The gradual cessation of the autonomous activity of the hypophysis, therefore, is manifested by gradually decreasing ovipositor growth.

(2) Substances with slowly increasing narcotic action, prolonged narcosis and subsequent slowly increasing narcosis produce a growth curve which may be similar to that of ovipositor growth, since the intensity of growth decreases as narcosis increases. These changes are displayed in the three above-mentioned narcotic actions related to the narcosis (see Fig. 6).

(3) A substance such as isobutane, isobutane, propane, or isobutane, produces complete narcosis. In the subsequent, rapidly increasing narcosis, the first phase of the narcosis (grades 3—1) are passed so quickly that the autonomous activity of the hypophysis cannot express itself adequately. Not until the second phase (starting when the gas is in the second phase) a phase in which only the autonomous activity of the hypophysis is resulting in ovipositor growth (see Fig. 11.) According to this view,

growth under the influence of alcohol would be expected, as shown in Fig. 5.

(4) When bitterlings are narcotized with alcohol or progesterone, and the narcotic state is ended quickly by the addition of fresh water, ovipositor growth will set in sooner and more markedly than when the fishes are left in the milieu mentioned (see Figs. 24 and 25). The accelerated cessation of the narcosis of the hypophysis, therefore, is manifested by earlier and more intensive ovipositor growth.

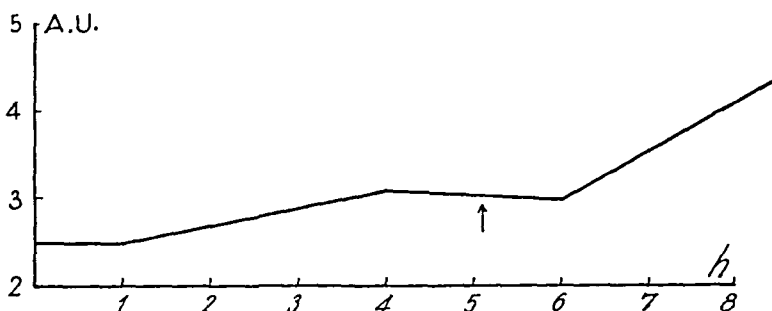


Fig. 24.

Ovipositor growth caused by ethanol. The arrow shows the moment when fresh water was added to the fishes.

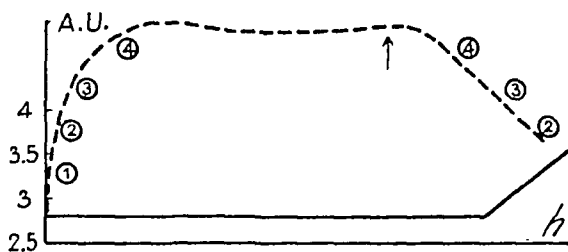


Fig. 25.

Ovipositor growth caused by a narcotizing quantity of progesterone. The arrow shows the moment when fresh water was added to the fishes.

(5) Sometimes a second narcotic wave may be observed after the first has passed off. In that case the growth-curve shows a period of lessened growth corresponding to a second increase in the depth of the narcosis (see Fig. 26, ethanol; Fig. 27, avertin).

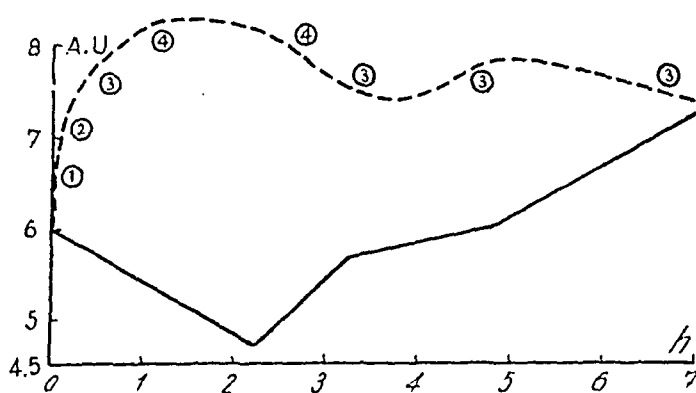


Fig. 26.

Reciprocal correlation between in- and decreasing depth of narcosis, respectively, and the rate of ovipositor growth after administration of ethanol.

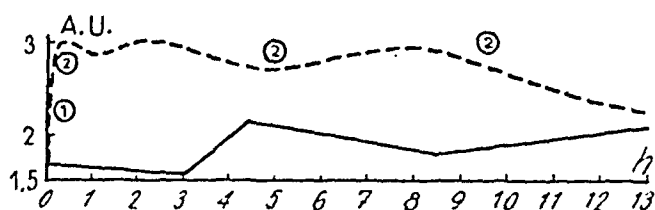


Fig. 27.

As fig. 26, but in respect of avertin.

(6) We have not, so far, found any substances producing narcosis with no subsequent ovipositor growth.

The chain-reaction set up by the action of narcotics on the female bitterling is illustrated by the schematic diagram in Fig. 28.

According to *Van Koersveld* (1947), the artificial induction of ovipositor growth is merely the simple manifestation of the damaging influence of any toxic agent. It will readily be seen from the foregoing discussion that this conception is untenable.

## SUMMARY

Artificial ovipositor growth can be induced in the bitterling by heat-shock, strong light, steroids and non-steroid narcotics.



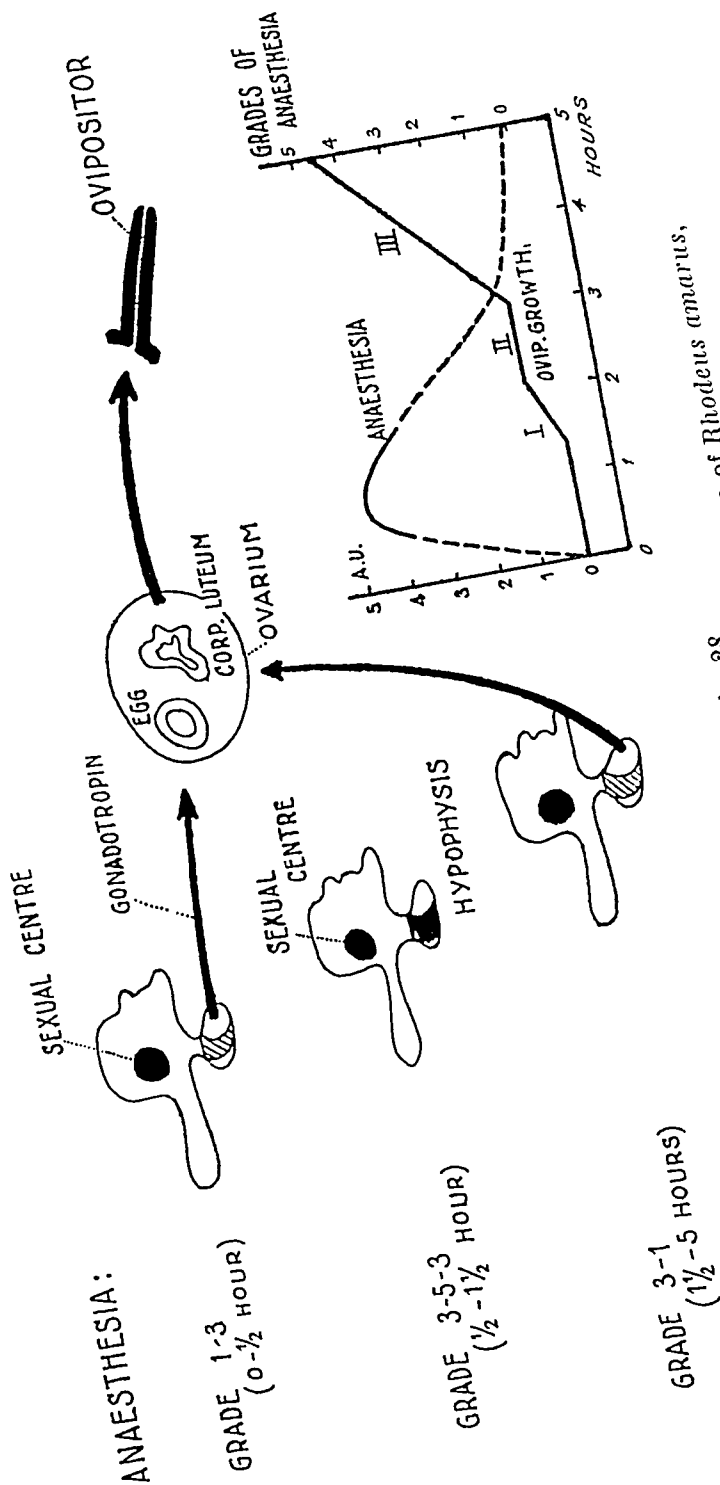


Fig. 28.  
Action of narcotics on the central nervous system of *Rhodnius amarus*.

the secretion

according to the »disinhibition hypothesis«.

hypophysis in the secretion

according to the »disinhibition hypothesis«.

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Some damaging agents investigated, e. g. mercuric chloride, antistin, trauma and cold-shock, do not produce ovipositor growth. Artificially induced ovipositor growth, therefore, is not a result of non-specific, noxious stimuli in general, as has been erroneously postulated by *Van Koersveld*.

Although in the ovipositor test, both steroid and non-steroid narcotics produce ovipositor growth, there is this remarkable difference between steroids and non-steroid narcotics, that, with the former (especially the pregnanes) the doses which only just produce ovipositor growth are many times smaller than the doses which only just produce narcosis, whereas in the case of the non-steroid narcotics, this margin is either small or reduced to zero.

Substances producing only narcosis but no ovipositor growth have not so far been found. The capacity of the substances mentioned to induce ovipositor growth may be explained by assuming that they block a »sexual centre« superimposed on the hypophysis, which causes the hypophysis to exert an autonomous activity.

The marginal difference between the doses inducing ovipositor growth and narcosis, of the steroids and the non-steroid narcotics respectively, is explained by assuming that steroids have a much greater affinity for the sexual centre than the non-steroid narcotics. By virtue of this fact, the steroids, and particularly the pregnanes, occupy a special place among those substances which induce ovipositor growth.

#### *Acknowledgements.*

We are greatly indebted to Professor *H. Selye* for reading the manuscript, to Dr. *W. S. Bullough* for his assistance and valuable advice during the preparation of this publication and to Miss *A. Hoetink* for the final preparation of the manuscript.

We wish to express our gratitude to the *Jan Dekker Foundation* and the *Hector Treub Fund* for their financial assistance.

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(Professor G. J. van Oordt, Ph.D.)

## ON THE ARTIFICIAL INDUCTION OF OVIPOSITOR GROWTH IN THE BITTERLING (RHODEUS AMARUS BL.)

### III. THE RELATION BETWEEN ARTIFICIALLY INDUCED OVIPOSITOR GROWTH AND THE ADAPTATION SYNDROME OF *SELYE*

BY

B. DE GROOT and J. J. DUYVENÉ DE WIT<sup>1)</sup>

#### INTRODUCTION

As the mammalian steroid hormones do not act directly on the ovipositor of the bitterling, and as their presence in the gonads of fishes has not so far been definitely demonstrated, we are of the opinion that their (indirect) action on the hypophysis of the bitterling is of a pharmacological nature. We may accordingly suppose that these substances act on the organism of the bitterling more or less as damaging agents, and that there are certain physiological mechanisms which help to produce a resistance to them. As shown in the preceding paper (*de Groot & Duyvené de Wit, 1949 b*), certain steroids and non-steroid narcotics as well as physical stimuli cause ovipositor growth, and the question therefore arises

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<sup>1)</sup> 33rd Communication of the »Werkgemeenschap voor Endocrinologie«.

whether such growth can be regarded as a concomitant phenomenon of the adaptation syndrome described by *Selye*.

According to *Selye* (1947, p. 837) »there are certain physiological mechanisms which help to raise resistance to damage as such«. »The sum of all those non-specific systemic reactions of the body which ensue upon long-continued exposure to stress has been termed the *general-adaptation-syndrome*. It is characterized by a number of morphologic and functional changes. Among the most prominent of these are: enlargement of the adrenal cortex with increased corticoid-hormone secretion, involution of the thymus and of other lymphatic organs, gastrointestinal ulcers, certain metabolic changes and variations in the resistance of the organism.

If an individual is continuously exposed to stress, the resulting general-adaptation-syndrome evolves in three distinct stages:

(1) *The alarm-reaction*, which is defined as the sum of all non-specific systemic phenomena elicited by sudden exposure to stimuli to which the organism is quantitatively or qualitatively not adapted«. »Usually, the alarm-reaction evolves in two distinct phases, the phenomena of shock being followed by those of counter-shock.

(2) *The stage of resistance* is defined as the sum of all non-specific systemic reactions elicited by prolonged exposure to stimuli to which the organism has acquired adaptation«. »During the stage of resistance, adaptation to one agent is acquired at the expense of resistance to other agents.

(3) *The stage of exhaustion* represents the sum of all non-specific systemic reactions which ultimately develop as the result of very prolonged exposure to stimuli to which adaptation had been developed, but could no longer be maintained«.

The three stages of the general adaptation syndrome are illustrated in Fig. 1, based upon measurable variations in resistance to damage.

By *specific resistance*, *Selye* (1947, p. 838) means »that type of injurement which increases resistance only against the particular type of stress to which the body had been ex-

posed; conversely, *non-specific resistance* designates the ability of the body to withstand a type of stress qualitatively different from that to which it had become adapted.

The term *adaptation energy* is used to describe the ability of the organism to acquire resistance to stress.

By definition, any agent capable of producing an alarm reaction is an *alarming stimulus*. Agents which »evoked intensive adaptive responses produce severe alarm-reaction symptoms«.

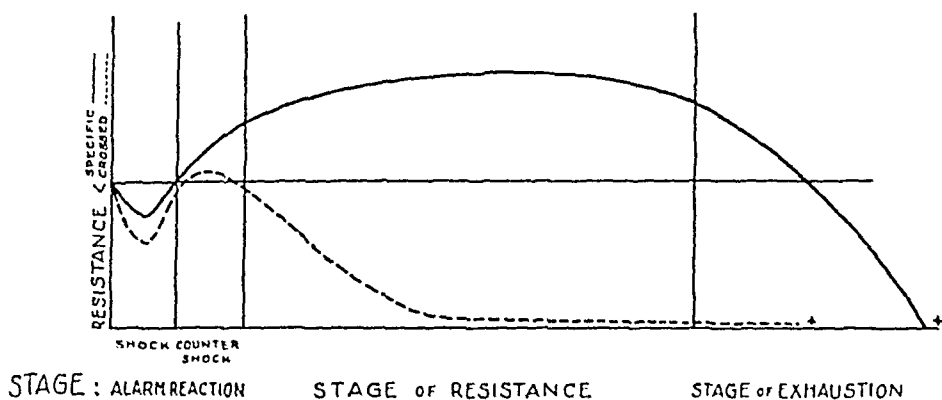


Fig. 1.

Schematic representation of the changes in specific (full line) and crossed (broken line) resistance during the three stages of the general-adaptation-syndrome.

(After Selye, 1946.)

In the bitterling, we think it hardly possible in view of difficulties of technical nature, to find any enlargement of the adrenal cortex or involution of the thymus and of other lymphatic organs as a result of the action of an alarming agent. We shall therefore confine ourselves to enquiring whether the terminology used by Selye can readily be applied to those changes in the growth of the ovipositor which occur after the repeated administration of a series of damaging agents.

## DISCUSSION

In the preceding paper (de Groot & Duyvené de Wit, 1949 a) an experiment was described in which, on 10 successive days and for 6 hours per day, 4  $\gamma$  progesterone, plus a

slight temperature-shock of 17—23° C. was given to a number of unsensitized fishes.

As Fig. 6 (1949 *a*, p. 260) shows, ovipositor growth was obtained on the first day, from an initial length of 0.5 A. U. On the third testing day, we find a greater starting length (3.3 A. U.), a longer latent period, a slightly shorter period of linear growth, and a somewhat smaller quantitative result (expressed in A. U.) at the end of the first period of linear growth.

In the preceding paper (*de Groot & Duyvené de Wit*, 1949 *b*) we expressed the opinion that steroids exert a special effect on a certain part of the central nervous system, which we have named the sexual centre. It might be possible that this centre, when stimulated repeatedly by a given quantity of progesterone, acquires an ever greater power of resistance against this damaging agent. The continuation of the damaging stimulus, however, must then lead to the gradual decrease of the resistance until a state of complete exhaustion is reached.

To demonstrate this, a number of fishes were exposed daily to narcotizing quantities of progesterone, at a constant temperature of 23° C. The results are given in Table 1. It will be seen that in the series treated with 200 and 300  $\gamma$  progesterone on the first testing day, the fishes had only slight resistance to the narcotic action of this substance. On the second day resistance was greater, and on the third day weak again. After a new peak on the fourth day, resistance gradually disappeared. This table also shows that in general a small initial ovipositor growth and a long duration of the first period of linear growth constitute the expression of weak resistance of the fishes to the stimulus administered.

It is now possible to interpret the experimental results given in our preceding paper (1949 *a*) with the aid of *Selye's* terminology:

(1) It is seen as a manifestation of heightened resistance, so that up to the fourth testing day (Fig. 6, p. 260) the initial length and the latent period have increased, whereas the period of linear growth and the total quantitative growth have de-

Table 1.

Dose progesterone per 750 ml water	Day of experiment	Average initial ovipositor length (A. U.)	Latent time (hours)	Quantitative increase in A. U.	Depth of narcosis (degrees 0-5)
200 $\gamma$	1	0.3	1.25	2.8	2.4
	2	4.2	2.0	3.2	1.8
	3	2.2	1.0	4.8	2.4
	4	4.3	2.0	2.5	1.8
	5	3.5	2.5	2.8	2.0
	6	2.3	2.25	4.0	2.4
300 $\gamma$	1	0.3	1.25	3.0	3.8
	2	7.5	4.0	0.8	2.5
	3	4.5	3.5	3.0	
	4	5.3	2.0	2.2	4.2
	5	5.5	1.75	1.5	3.3
	6	5.3	2.75	2.2	3.8

creased. After resistance has reached its maximum on the fourth testing day, there is increasing exhaustion during the fifth and sixth days. On the seventh and eighth testing days, recovery sets in again, but the adaptation energy is evidently insufficient to reach the same degree of resistance as on the fourth day; on the ninth day resistance once again decreases.

Fig. 7 (p. 262) shows still more clearly how the animal's efforts at adaptation spread wave-like over the ten testing days. The variations in the initial length — as a measure of the quantity of adaptation-energy present in the sexual centre — become even smaller, which would seem to point to the beginning of the exhaustion stage (cf. also Figs. 8 and 9, p. 263).

(2) Fig. 10, p. 264, refers to fishes which, without heat-shock, were given exclusively 4  $\gamma$  progesterone and were kept in water at 23° C. During the first two days, the starting length increases very markedly, which may be regarded as a sign of the rapid acquisition of high resistance. But as a result of the harmfulness of the un-physiologically high temperature, greater exhaustion sets in than after the experiment described in (1) above, in which the fishes, after the experiment was ended, were kept in water at 17° C. for 18 hours out of the 24.



(3) When fishes sensitized with luteidin are given 8  $\gamma$  progesterone, we obtain a curve with a small initial length, short latent period and large ovipositor growth within a short time. Owing to the pre-treatment with luteidin, the specific resistance against luteidin is greatly raised, but that against progesterone evidently greatly reduced. This fact is in agreement with *Selye's* observations in mammals.

(4) The influence of heat-shock. In the daily repeated tests with progesterone, described in our preceding paper (1949 a, p. 260) certain curves are noticeable showing a great initial length. The latent period, however, is shorter than was expected, and during this period the length of the ovipositor is reduced considerably. This effect of the heat-shock appears more particularly in fishes which have a strong resistance to progesterone. It may also be observed in the control-fishes, but in a lesser degree. The action of both stimuli together, therefore, is not the same as the sum of the separate actions. This is also seen if 8  $\gamma$  progesterone plus a heat-shock are administered to fishes previously sensitized by luteidin.

This is in accordance with what was said in (3) above. Generally speaking, therefore, it appears that stronger specific resistance is coupled with reduced non-specific resistance (*Selye*, 1947).

The effect of the heat-shock is different on the first testing days of each series. In the first place, there is ovipositor growth instead of a decrease in the length of the ovipositor (cf. Figs. 2 and 3); in the second place, the effect of both stimuli together is equal to the sum of the separately observed effects (fig. 3). In this case, therefore, the fishes have not yet reacted to the un-physiological stimuli, and their adaptation energy is sufficient to cope quantitatively with both stimuli. The growth-curve does not show a latent period under the influence of the heat-shock, which might be an indication that, in this case, hyper- and hypotension — well known as rapidly developing shock-phenomena — here play a rôle. Ovipositor growth might then be a consequence of blood- and lymph-congestion in the inter-epidermal lacunae of the ovipositor.

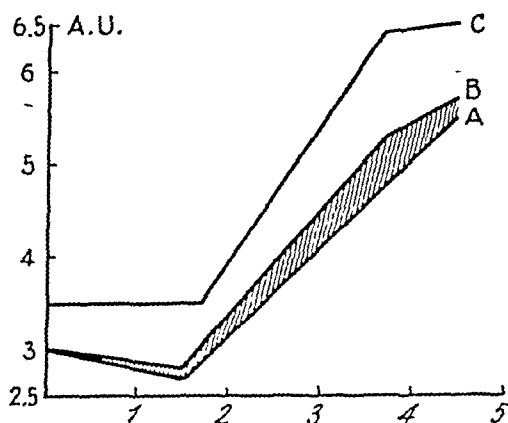


Fig. 2.

A = ovipositor growth after administration of progesterone plus heat shock, in fishes that have already reacted a few times to these stimuli.

B = A, less the separately observed effect of the heat shock.

C = progesterone without heat shock, found empirically.

B (theoretical) and C (empirical) show a considerable difference.

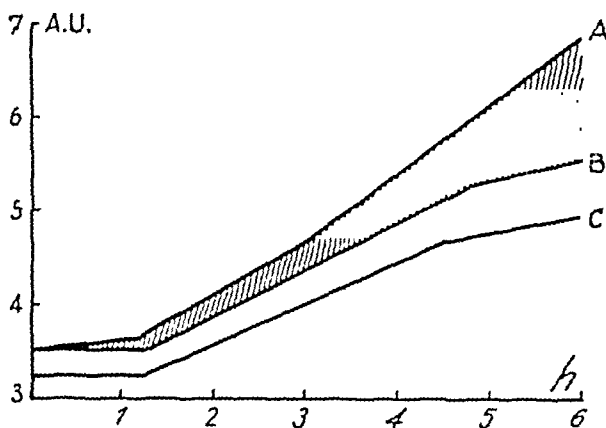


Fig. 3.

A = ovipositor growth after administration of progesterone plus heat shock, in fishes reacting to these stimuli for the first time.

B = A, less the separately observed effect of the heat shock.

C = progesterone without heat shock; the fishes had been adapted.

B (theoretical) and C (empirical) are practically identical.

(5) The influence of the heat-shock was shown, in a series of experiments with pregnenolone, to be much greater than in a series given progesterone in the same dose. Pregnenolone

therefore required, in this respect, more adaptation energy than progesterone.

We can thus conclude from the foregoing that ovipositor growth, under certain circumstances, can be interpreted as a concomitant phenomenon of the adaptation syndrome, described by *Selye*. So long as the effectiveness of ovipositor growth as means of defence against damaging agents cannot be proved, we are not justified in regarding it as an essential part of the adaptation syndrome.

It appears that not every adaptation syndrome is coupled with ovipositor growth. The following may be distinguished:

- (1) adaptation to the repeated action of steroids, non-steroid narcotics, strong light, and heat, coupled with ovipositor growth;
- (2) adaptation to the repeated action of non-steroid toxic substances, cold, or trauma, without ovipositor growth;
- (3) ovipositor growth which is not a result of adaptation to damaging agents. This occurs under natural conditions during the spawning season. Although all reactions to the milieu may be regarded as adaptative phenomena, and although it is often difficult to draw a distinct line between natural and unnatural stimuli, one would hardly be justified in such circumstances in speaking of an adaptation syndrome in the extreme sense indicated by *Selye*.

The experiments, described in this and the foregoing two papers, were carried out independently before the publication of *van Koersveld's* thesis (1949). They will be continued with reference to this publication.

### SUMMARY

The growth of the ovipositor of the bitterling (*Rhodeus amarus* Bl.), resulting from the influence of repeated administration of certain kinds of chemical and physical stimuli, may readily be explained with the aid of *Selye's* terminology of the adaptation syndrome. In agreement with this view, artificially induced ovipositor growth may under special circum-

stances be regarded as a concomitant phenomenon of an adaptation syndrome. Nevertheless, not every adaptation syndrome is coupled with ovipositor growth.

#### *Acknowledgments.*

We are greatly indebted to Professor *H. Selye* for reading the manuscript, to Dr. *W. S. Bullough* for his assistance and valuable advice during the preparation of this publication and to Miss *A. Hoetink* for the final preparation of the manuscript.

We wish to express our gratitude to the *Jan Dekker Foundation* and the *Hector Treub Fund* for their financial assistance.

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# ANNOUNCEMENTS

## from the Endocrinological Societies

### DANISH SOCIETY FOR ENDOCRINOLOGY

15. Meeting, September 22, 1949, Zoölogical Museum, Copenhagen.

*Knud Lundbæk*: The later stages of diabetes mellitus.

*Paul Horstmann*: The sexual hormones in diabetes mellitus.

16. Meeting, December 14, 1949, Domus medica, Copenhagen.

*Choh Hao Li* (guest): The adrenocorticotropic hormone (ACTH) of the anterior hypophysis.

From the Endocrinological Division of the Department of Medicine of Serafimerlasarettet, Stockholm, and the Biochemical Institute of the University of Uppsala, Sweden.

## RESULTS OF ADMINISTRATION OF ADRENOCORTICOTROPHICALLY ACTIVE PEPTIDES (ACTH PEPTIDES) TO A PATIENT SUFFERING FROM RHEUMATOID ARTHRITIS<sup>1)</sup>

BY

ROLF LUFT, BJÖRN SJÖGREN and CHOH HAO LI<sup>2)</sup>

It has been demonstrated by one of us (Li, 1948) that a certain peptic digest of adrenocorticotrophic hormone (ACTH) retains the biological activity, and that the adrenocorticotrophic potency resides in the peptide residues. The present report concerns the effect of the adrenocorticotrophically active peptides (ACTH peptides) on a patient suffering from rheumatoid arthritis.

### MATERIAL AND METHODS

Pure ACTH was isolated from sheep pituitary glands by a method previously described (Li *et al.*, 1942—43). The ACTH peptide preparation was obtained by pepsin digestion as de-

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<sup>1)</sup> This study was aided by a grant from Statens Medicinska Forskningsråd (The Medical Research Council of Sweden).

<sup>2)</sup> On sabbatical leave from the University of California, Berkeley, California.

scribed by *Li* (1948). The protein hormone or the peptide mixture were dissolved in physiological saline. The daily dose was divided into six injections; each injection consisting of one ml. The patient was thus injected every four hours. The hormone solutions were stored in the refrigerator when not in use.

The determinations of *sodium* and *potassium* in urine were performed with the Beckman flame spectrophotometer. The *chlorides* in urine were determined according to *Bang-Larsson*. *Urinary 17-ketosteroids* were assayed by *Hamburger's* micro-method (*Hamburger & Rasch*, 1948). *Uric acid* was determined by the uricase method of *Praetorius* (1949). *Total nitrogen* in urine was determined according to *Kjeldahl*,<sup>1)</sup> phosphorus according to *Youngburg & Youngburg* (1930), calcium by a modification of the *Kramer-Tisdall* method. The *eosinophil leucocytes* were determined according to *Rud* (1947).

### CASE RECORD

C. O. B. H., 58 years old married man. In 1922 the first signs of rheumatoid arthritis appeared. Now and then he had pains from the joints, but severe changes and symptoms did not appear until spring 1949. When admitted to the hospital in April 1949 he showed a typical rheumatoid arthritis in advanced stage. There were pains, stiffness, and swellings of the following joints: hands, feet, knees, and shoulders. The patient showed a moderate rise of temperature, hypochromic anemia with a low serum iron content and a low protein content in serum. He also had a rheumatic valvular heart disorder. Despite treatment with gold salts, chemotherapy, administration of iron intravenously, and physical therapy the condition of the patient deteriorated markedly. Just before the onset of ACTH treatment the patient was unable to move his arms, hands, feet, and neck. Because of arthritic changes in the mandibular joints he could hardly swallow anything but fluid or minced food. He had an evening tem-

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<sup>1)</sup> We are indebted to professor Erik Jorges for the nitrogen determinations.

perature of over 39° Celcius. The sedimentation rate was consistently above 100 mm. per hour.

*The patient was put on a constant diet during the whole experiment.* After a control period he received for four days (between November 6 and 10) a daily dose of 25 mg. of pure ACTH protein. This period was followed by a control period of eight days. He then received for another four days period (between November 18 and 22) a daily dose of 13 mg. of ACTH peptides. After another control period of six days the patient finally was injected with 13 mg. of pure ACTH protein daily for five days (between November 28 and December 3), followed by a control period of eight days.

## RESULTS<sup>1)</sup>

### *Clinical data.*

The clinical improvement was essentially the same after the administration of either ACTH protein or ACTH peptides. It was evident that an improvement occurred within three hours after the first injection. The patient began to sweat, the rectal temperature showed a definite fall (see Fig. 1). The pains and stiffness of the joints gradually disappeared during the first day of treatment. On the second day the swelling of the joints had markedly decreased and the patient could even get out of bed and sit with comparative ease.

Six to eight hours after the last injection the symptoms of the patient began to reappear with an accompanied rise of temperature. The condition of the patient returned to that of the pretreatment period within 24 hours.

### *Sedimentation rate (Fig. 1).*

Each period of treatment was followed by a marked decrease of the sedimentation rate. However, the change did not appear in the first day of injection nor did the sedimentation rate rise immediately after end of treatment.

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<sup>1)</sup> Similar results in a patient with rheumatoid arthritis have been obtained by Kinsell & Li (to be published). They administered a daily dose of 100 mg. ACTH peptide. No comparison was made with the pure ACTH protein.



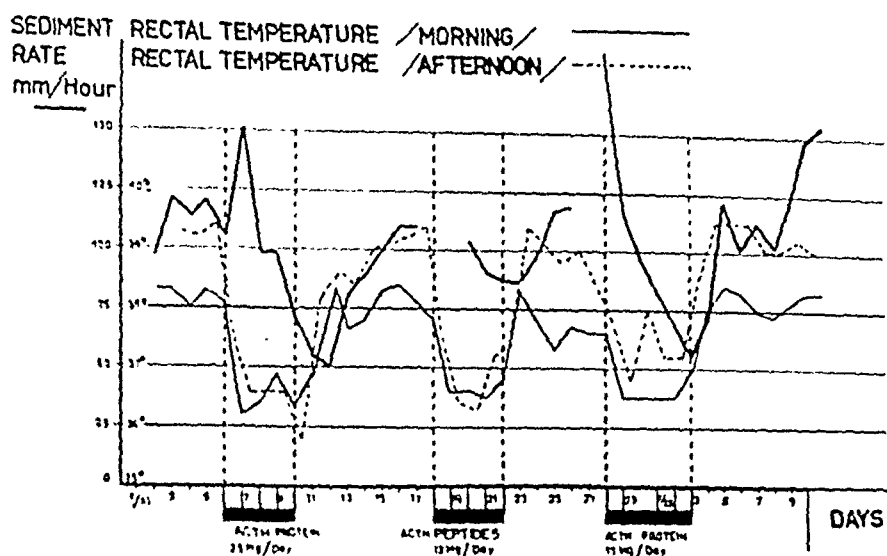


Fig. 1.  
Rectal temperature and sedimentation rate.

### *Eosinophil leucocytes* (Fig. 2).

In the control period, before the administration of ACTH peptides, the eosinophilic count was approximately 150 per  $\text{mm}^3$ . This decreased to almost zero on the first day of in-

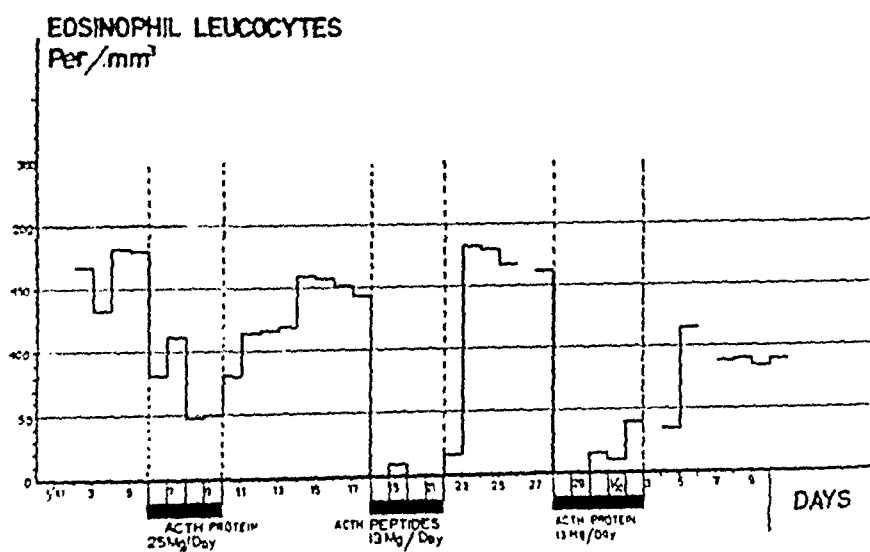
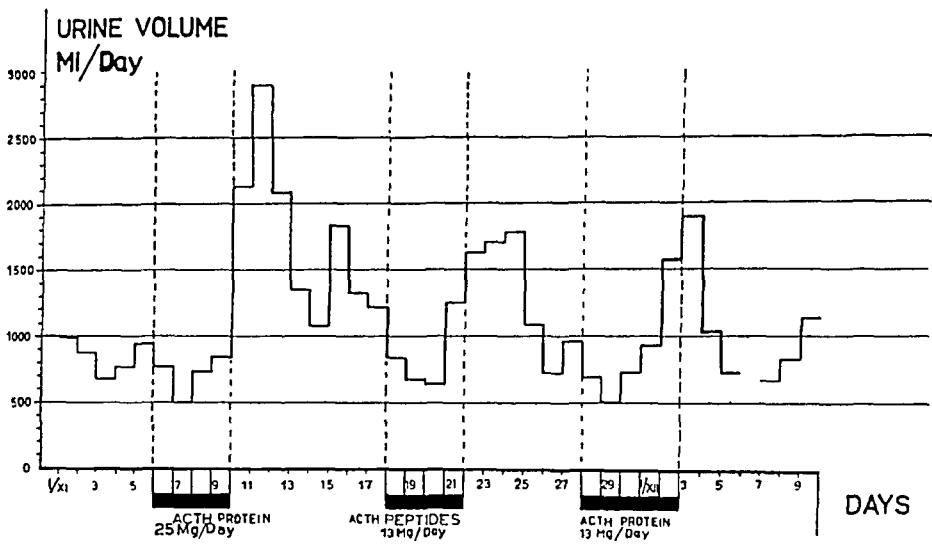


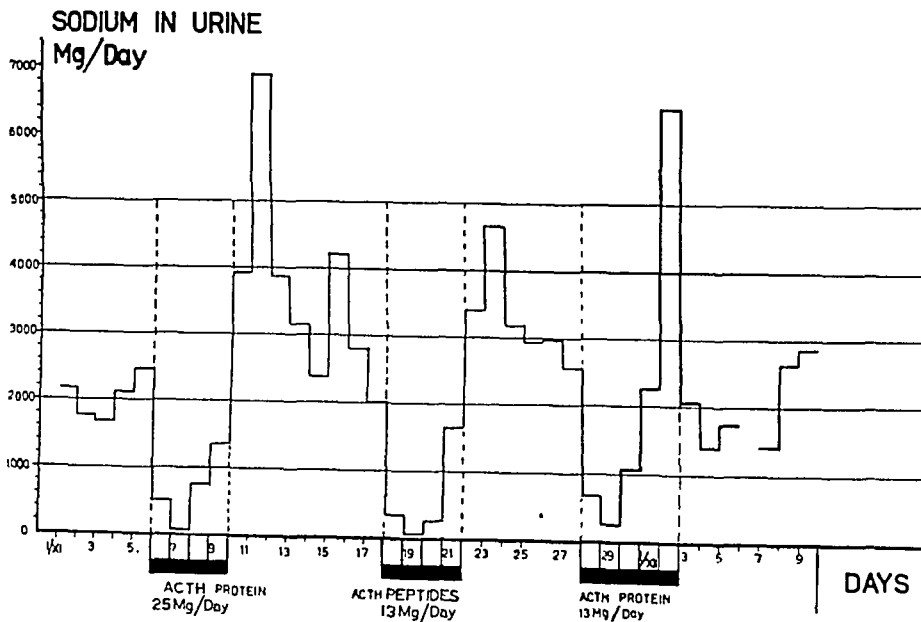
Fig. 2.  
Number of eosinophil leucocytes.

*Urine volume (Fig. 3).*



*Fig. 3.*  
Volume of urine.

jection and was maintained at zero level through the period of peptide administration. When the ACTH peptides were withdrawn it was followed by a rapid rise of the eosinophilic



*Fig. 4.*  
Amount of sodium in the urine.

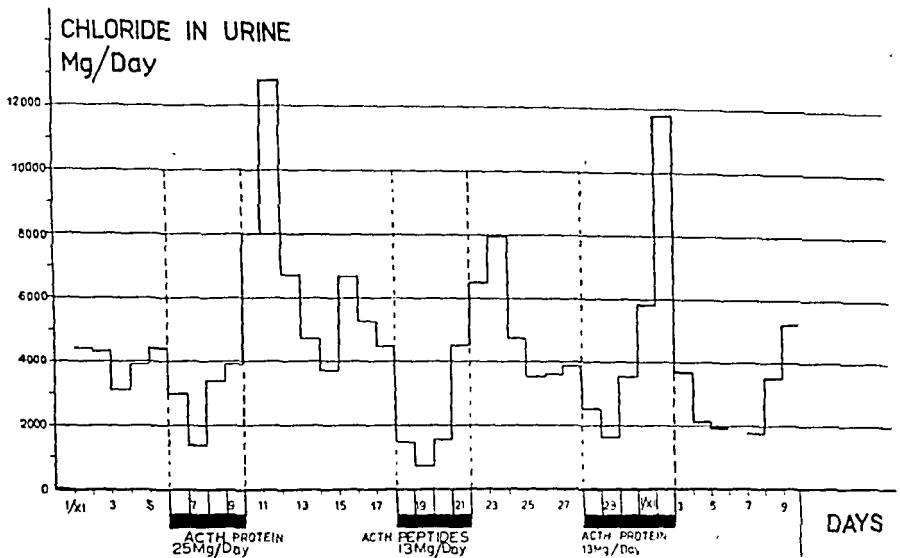


Fig. 5.

Amount of chloride in the urine.

count to almost 180 per  $\text{mm}^3$ . Similar results were obtained with ACTH protein.

In every period of treatment there was a decrease of urine volume during the first three days followed by a rise to a value higher than that of the pretreatment period.

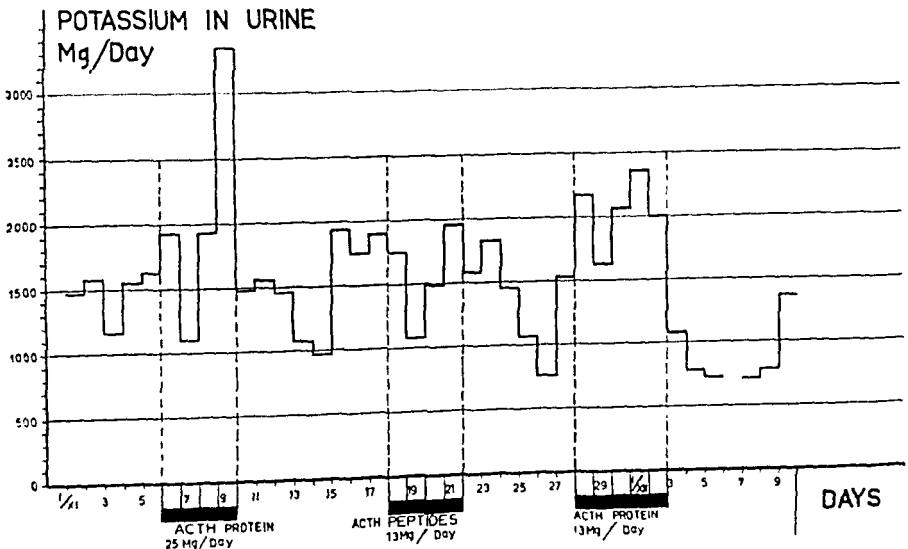


Fig. 6.

Amount of potassium in the urine.

*Sodium and chloride excretion (Figs. 4 and 5).*

The retention of sodium was evident in the first three days of treatment. The sodium content of the urine fell markedly from about 2000 mg. per day to less than 100 mg. per day after the patient had received 26 mg. of peptides during the first two days. There was then a sudden rise of sodium excretion when the administration stopped.

The changes of the chloride excretion followed the same pattern as the sodium (see Fig. 5).

*Potassium excretion (Fig. 6).*

The excretion of potassium showed rather inconsistent changes. However, there was a tendency to increased excretion during the hormone administration.

*Calcium excretion (Fig. 7).*

In the first periods of administration of ACTH protein and ACTH peptides the urinary calcium level was fluctuating markedly and no conclusions could be drawn. When the ACTH protein was used after the peptide period a significant rise of calcium excretion occurred towards the end of the injection period.

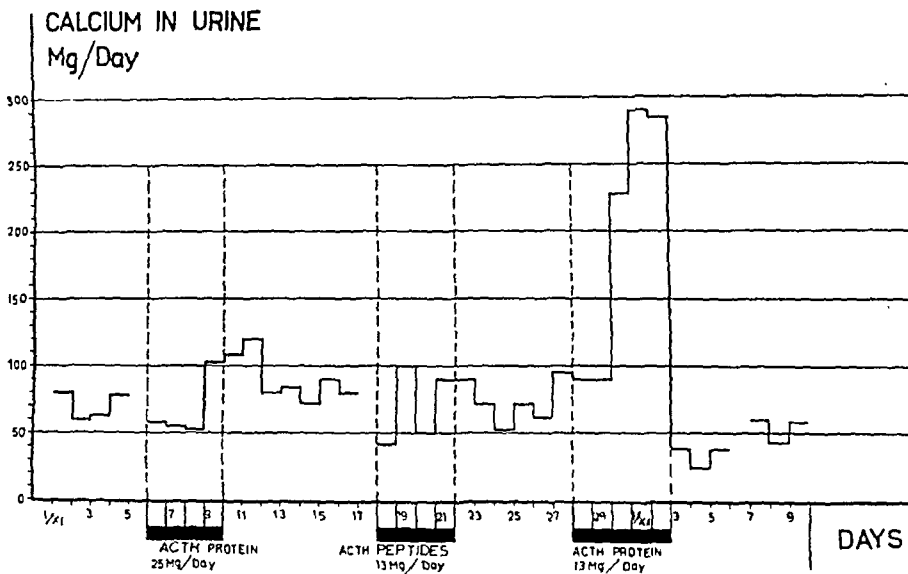
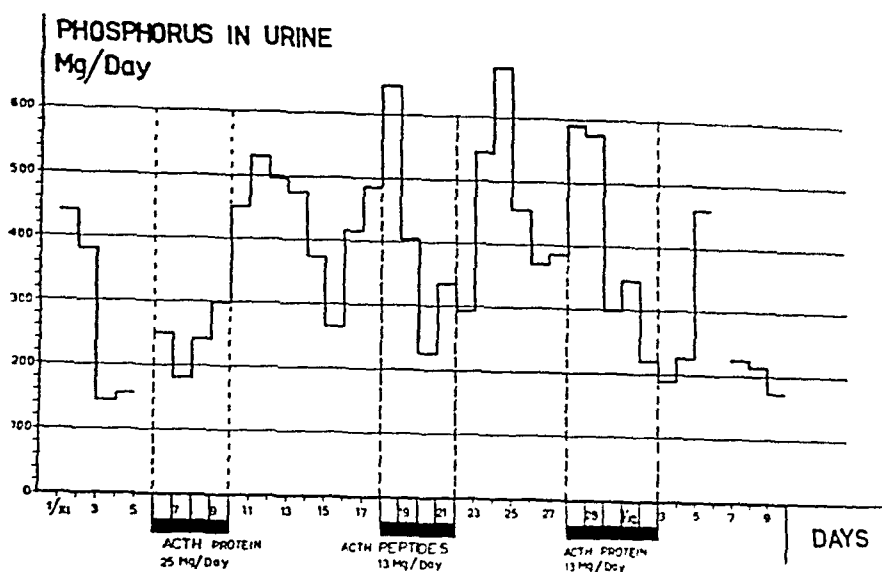
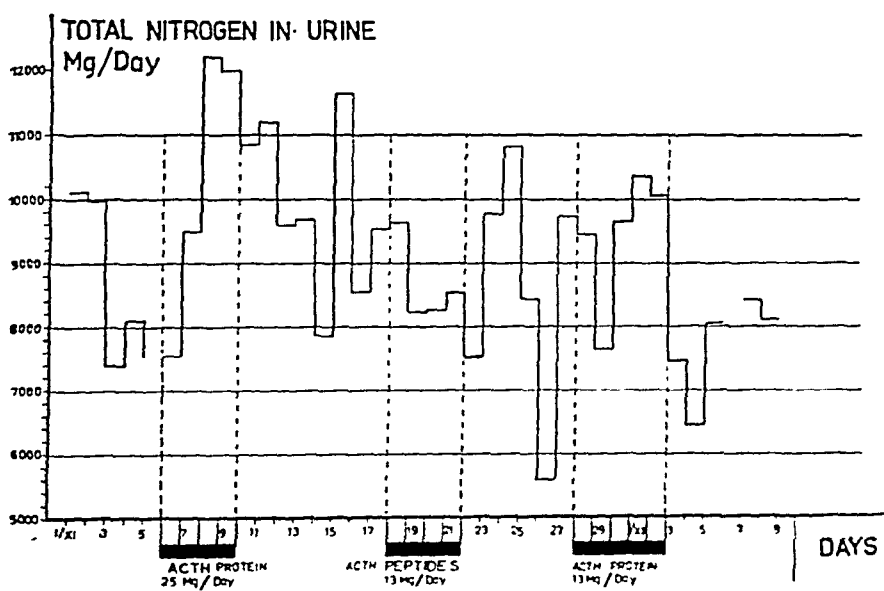


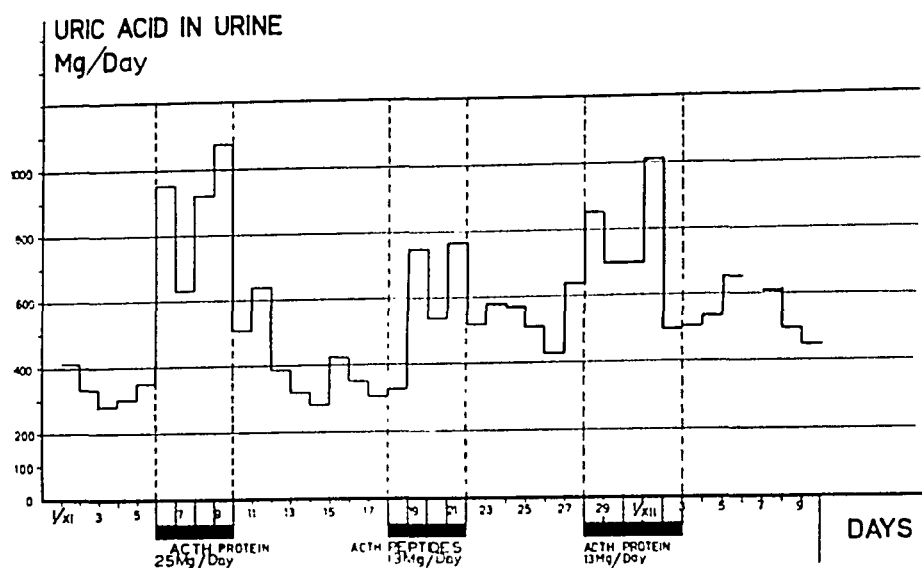
Fig. 7.  
Amount of calcium in the urine.



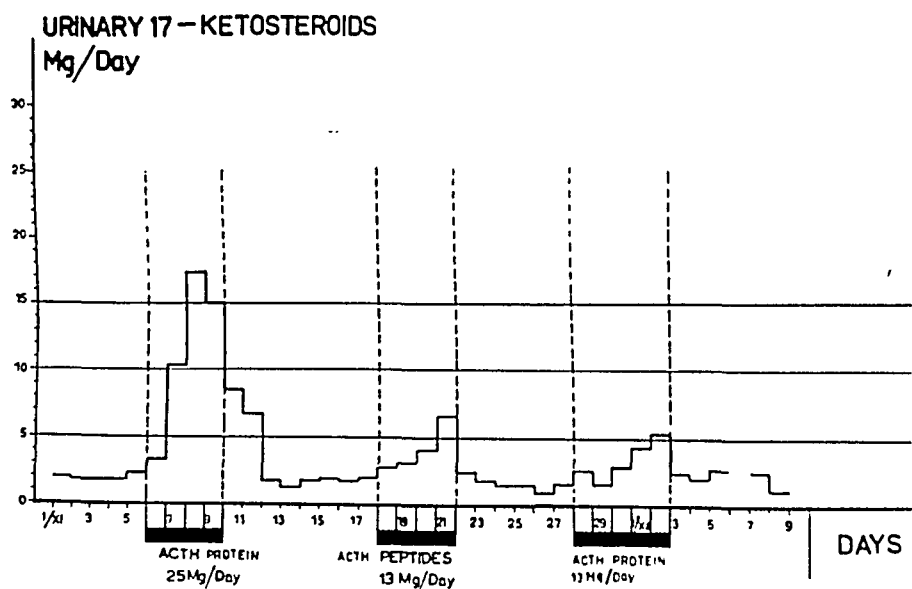
*Fig. 8.*  
Amount of the phosphorus in the urine.



*Fig. 9.*  
Amount of total nitrogen in the urine.



*Fig. 10.*  
Amount of uric acid in the urine.



*Fig. 11.*  
Urinary excretion of 17-ketosteroids.

*Phosphorus and total nitrogen in urine* (Figs. 8 and 9).

The changes of both phosphorus and nitrogen content in the urine during the administration of 13 mg. of ACTH peptides or 13 mg. of ACTH protein daily were irregular and make any evaluation difficult. But, when 25 mg. of ACTH protein were injected daily there was a rise of total nitrogen in urine. It may be that a significant change will occur if the injections are extended to a longer period of time.

*Uric acid excretion* (Fig. 10).

On the second day of ACTH peptides administration the amount of uric acid in the urine increased from approximately 300 mg. to more than 700 mg. per day. Immediate fall of uric acid excretion occurred when the ACTH peptides were withdrawn. Similar results were obtained by the administration of ACTH protein.

*Excretion of 17-ketosteroids* (Fig. 11).

The 17-ketosteroid excretion in the control periods was consistently low and had a level of 2 mg. per day or less. When the ACTH peptides were administered the concentration of the urinary 17-ketosteroids increased gradually to a maximum of 7 mg. a day. Similar patterns were obtained using the same dose of ACTH protein. However, if the daily dose of ACTH protein was increased to 25 mg. per day, the excretion of 17-ketosteroids increased sharply from 2 mg. to 17 mg. per day. As soon as the hormone injections stopped, the steroid excretion returned to the pretreatment level.

## SUMMARY

It was found that ACTH peptides were beneficial in a patient — a male, 58 years old — suffering from severe rheumatoid arthritis using a daily dose of only 13 mg. The improvement was evident within three to four hours after an injection of 2 mg. of the peptides. A similar beneficial effect was also observed when pure ACTH protein was used.

There were no differences between ACTH peptides and ACTH protein in their ability to stimulate the adrenal cortex as shown by the retention of sodium, chlorides and water. by the increase of the excretion of 17-ketosteroids and uric acid, and by the disappearance of circulating eosinophil leucocytes.

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From the State Serum Institute, Copenhagen, and the  
Pediatric Department of Rigshospitalet, Copenhagen.

## FALL IN HYALURONIDASE-INHIBITION IN SERUM DURING ADMINISTRATION OF ADRENOCORTICOTROPIC HORMONE (ACTH)\*)

BY

KAI SCHMITH and VIGGO FABER

Most normal sera and those from patients with different diseases contain a non-specific inhibitor of hyaluronidase (*Adner, 1948, Dorfman, Ott & Whitney, 1948, Hadidian & Pirie, 1948, McClean, 1942*). It is thermostable and acts on all kinds of hyaluronidase. *McClean (1942)* suggests, that the inhibitor is associated with the pseudoglobulin fraction, while *Glick & Moore (1948)* state that it migrates chiefly with the albumin fraction by electrophoresis. Sera of different origin vary greatly in their ability to inhibit the same enzyme (*McClean, 1942*).

*Glick and co-workers (Glick & Campbell, 1948, Glick & Gollan, 1948, Grais & Glick, 1948)* have observed a rise in the inhibitory capacity in sera from monkeys with experimental poliomyelitis and sera from patients with different diseases.

When hyaluronidases are used as antigens, *specific* anti-hyaluronidases, antagonistic to the homologous enzyme are

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\*) This investigation is part of a study carried out with the aid of grants from Rigsforeningen til Bekæmpelse af rheumatiske Sygdomme, Kong Christian den 10's Fond and P. Carl Petersen's Fond.

produced. These antibodies are thought to occur in the  $\gamma$ -globulin fraction and are thermostable. (*Glick & Moore, 1948*). Recently we have reviewed the literature concerning these problems (*Schmith & Faber, 1949*).

During administration of ACTH to a patient with rheumatoid arthritis a fall in the non-specific hyaluronidase inhibition of the serum was observed.

### METHOD AND MATERIALS

In the determination of hyaluronidase a modified turbidimetric method was used. This method, first described by *Kass & Seastone (1944)*, and later modified (*Dorfman, Ott & Whitney, 1948*, *Meyer, 1947*, *Tolksdorf, McCready, McCullagh & Schwenk, 1949*), is based on the observation that pure hyaluronate at pH 4.2 gives a fairly stable colloidal suspension with diluted serum. The turbidity obtained is proportional to the quantity of hyaluronic acid. Hyaluronic acid depolymerized with hyaluronidase gives no turbidity.

The hyaluronic acid used was isolated as sodium hyaluronate from human umbilical cord as described by *Blix & Snellman (1945)*. Hyaluronidase was prepared from bovine testes by ammonium sulphate fractionation.

Acidified serum reagent: 10 rabbits were heart-punctured; the sera diluted 10-fold with 0.5 M acetate buffer pH 4.2 and the pH adjusted to 3.1 with 4 M hydrochloric acid.

With an automatic pipette 0.5 ml. of serial dilutions of a standard hyaluronidase, newly dissolved in 0.1 M acetate buffer pH 6 with 0.15 M sodium chloride, is placed in tubes. To each tube 0.5 ml. of the substrate containing 40 mg. per cent hyaluronic acid in 0.1 M acetate buffer pH 6 with 0.15 M sodium chloride, is added. The tubes are incubated for half an hour at 37° C. The enzyme is then inactivated by heating at 60° C for 10 minutes. To each tube are added 4 ml. of a mixture containing 3 parts 0.5 M buffer pH 4.2 and 1 part of the acidified serum reagent. After half an hour the turbidities

are read in a Coleman spectrophotometer using a wavelength of 580 m $\mu$ .

A standard curve (Fig. 1, A) for the hyaluronidase preparation is obtained by plotting the densities against the

FIG. 1

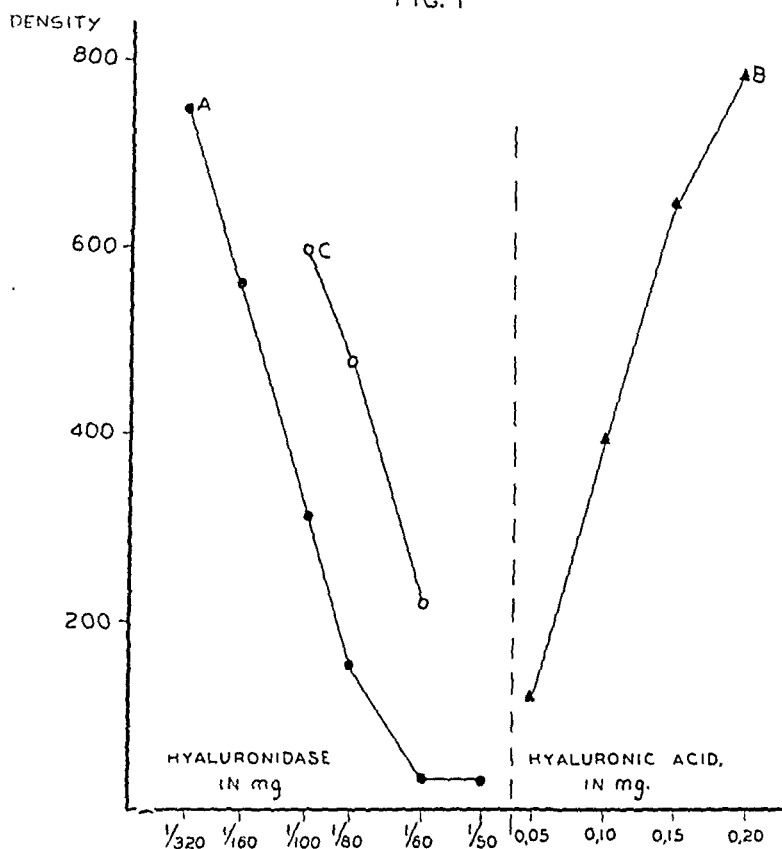


Fig. 1.

A: Standard curve for hyaluronidase.

B: Standard curve for hyaluronic acid.

C: Curve for hyaluronidase inhibited by serum.

amount of hyaluronidase, and a standard curve for hyaluronic acid by plotting densities against various amounts of hyaluronic acid (Fig. 1, B). The enzyme is expressed arbitrarily in turbidity reducing units (T. R. U.); one unit is defined as that amount of enzyme which reduces the turbidity given

by 0.2 mg. of hyaluronate to that given by 0.1 mg. (Meyer, 1947).

At first we found this method to be inaccurate; the tur-

FIG. 2

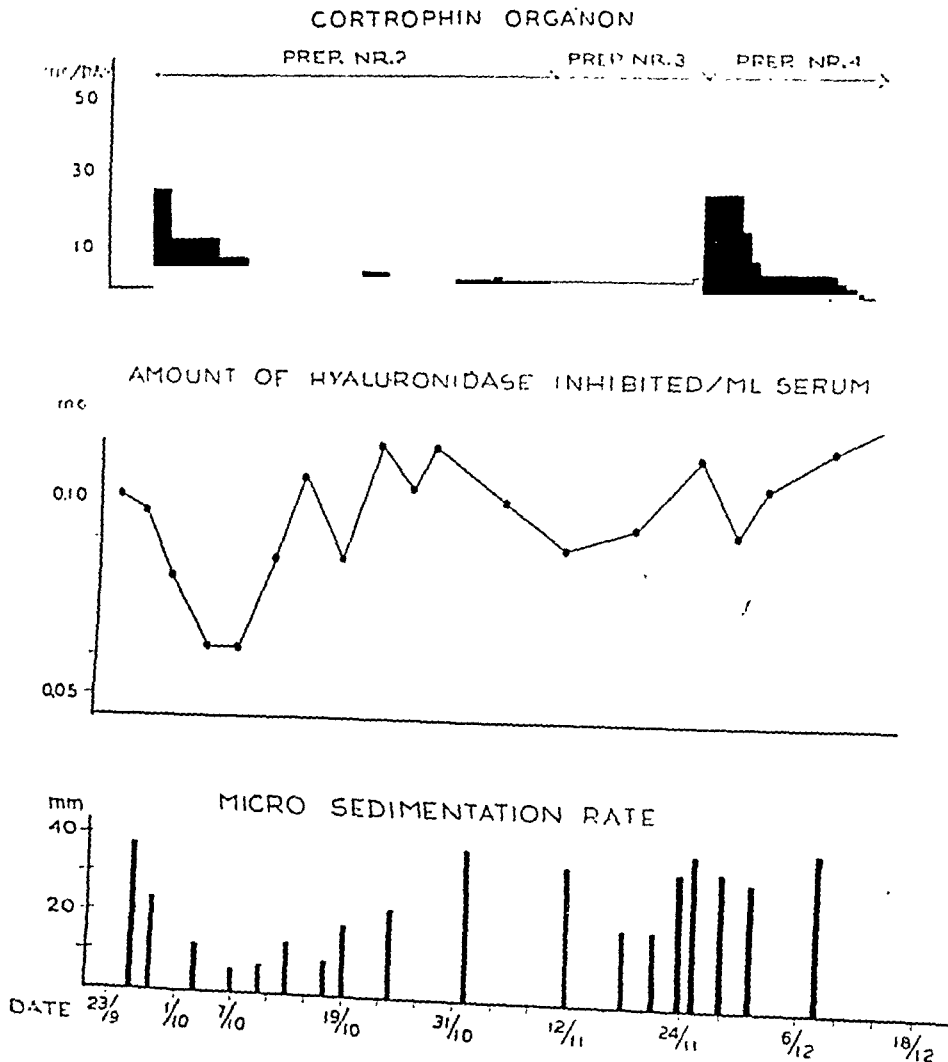


Fig. 2.

Variation in hyaluronidase inhibition and micro sedimentation rate during administration of ACTH (Cortrophin).

bidities were very low and the difference between the turbidity given by 0.2 and 0.1 mg. hyaluronic acid was slight. In agreement with *Tolksdorf et al.* (1949) we suspected this to be due

to the serum reagent. However, if the serum reagent is stored at a temperature of  $-15^{\circ}\text{C}$ , a gradual alteration occurs and the turbidities now obtained are greater and depend more on the concentration of hyaluronic acid; after 3—4 weeks, maximum turbidity occurs. If it is now kept at higher temperatures the turbidity values are gradually reduced. Results with this method are reproducible to approx.  $\pm 5$ —10 per cent.

Anti-hyaluronidase is determined in the following way: Test tubes with serial dilutions of hyaluronidase as described above, are incubated with 0.05 ml. serum or serum dilutions for 20 minutes at  $37^{\circ}\text{C}$ . Some of the enzyme is inhibited and consequently the turbidities obtained are higher and the curve displaced (Fig. 1, C). The displacement, which is read on the abscissa, corresponds to the amount of enzyme inhibited.

In this investigation all sera have been tested with the same stable bovine testes preparation and the inhibiting capacity is expressed in mg. hyaluronidase inhibited per ml. serum and not in units. (With the method here employed 1 mg. hyaluronidase was equal to 120 T. R. U.).

## RESULTS

The patient, a girl 10 years old, had suffered from rheumatoid arthritis from her second year. She had been treated for three periods with gold salts, but this and all other treatments were without effect. Administration of ACTH (Cortrophin Organon) commenced on the 26th of September and continued for  $2\frac{1}{2}$  months, with varying daily doses (Fig. 2). Three different batches of ACTH were used (No. 2, 3, and 4). The daily amount of ACTH was given as four injections in the course of the day.

The inhibitory capacity of the serum varied, but generally, with intensification of ACTH administration, a marked fall was noted. However, with preparation No. 4, presumably not so potent, there was only a slight fall in inhibitory capacity

(Fig. 2). During the administration of ACTH preparation No. 2 marked clinical improvement was noted; when preparation No. 4 was used the clinical improvement was not so great.

Sera, heated at 56° C for 20 minutes, lost their inhibiting property.

Sera before, during, and after administration of ACTH were also examined for antihyaluronidase antagonistic to streptococcal hyaluronidase (Lancefield's group A). None of the sera inhibited the streptococcal hyaluronidase.

## DISCUSSION

In various diseases, viz. rheumatic fever, rheumatoid arthritis, fibrositis, sclerodermia and myxoedema pathological lesions of the connective tissue are found. The mucopolysaccharide hyaluronic acid is an important constituent of the intercellular ground substance of connective tissue and is probably formed by the fibroblasts.

*Ragan & Meyer* (1949) found an incompletely polymerized hyaluronic acid present in the synovia in rheumatoid arthritis and suggest that the primary defect lies in an abnormal synthesis of hyaluronic acid and not in an action of hyaluronidase.

Joint lesions, resembling rheumatoid arthritis, occur in hormonal disturbances, e. g. some cases of thyrotoxicosis and Cushing's syndrome, overdose of desoxycorticosterone acetate in Addison patients and after administration of desoxycorticosterone acetate in rats (*Selye et al.*, 1944). These lesions may be explained by an influence on the formation of hyaluronic acid, which seems to be hormonally regulated. Thus, in myxoedema, the amount of hyaluronic acid is increased in skin and synovia (*Ropes et al.*, 1947), and oestrogen stimulation in monkeys produces a mucin-containing exudate in the sexual-skin (*Ogston et al.*, 1939), later a more generalized edema, (*Bachman et al.*, 1935).

Possibly ACTH and Cortisone also influence the hyaluronic acid, and the mechanism may be explained, either by an effect on the supposed abnormal synthesis of hyaluronic acid or by an inhibition of enzymatic hydrolysis of the hyaluronic acid. *Seifter, Baeder & Dervinis* (1949) have shown that ACTH, Cortisone and some cortisone-related gluco-corticoids have an anti-hyaluronidase effect.

It is difficult to reconcile these observations with the fall in the inhibitory effect of serum, which we have found.

### SUMMARY

In a case of rheumatoid arthritis administration of ACTH produced a fall in the serum's non-specific hyaluronidase-inhibiting capacity, paralleled by a marked clinical improvement.

Serum from the patient did not inhibit streptococcal hyaluronidase (Lancefield's group A).

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## THE EFFECT OF ACTH PROTEIN AND ACTH PEPTIDE ON THE HYALURONIDASE INHIBITOR OF HUMAN SERUM<sup>1</sup>)

### A PRELIMINARY REPORT

BY

ERICK Y. HAKANSON<sup>2</sup>) and ROLF LUFT

It has been shown that the hyaluronidase inhibiting property of blood serum is increased in various infections and rheumatoid diseases. This increase has been noted for the specific antibody-type of inhibitor (*Friou & Wenner*, 1947, *Thompson and Moses*, 1948, *Quinn*, 1948, *Harris & Harris*, 1949) as well as for the general non-specific one (*Glick & Gollan*, 1948, *Grais & Glick*, 1949). The increase has also been observed in cancer (*Hakanson & Glick*, 1948, *Fulton et al.*, 1948). The mechanism and significance of this observed increase of hyaluronidase inhibitor in various pathological conditions is at present unknown. Some of the highest values have been observed in rheumatoid conditions, and the variations of the inhibitor in these cases can be roughly correlated with the patient's clinical state, being highest during the acute stages of the disease (*Quinn*, 1948, *Grais & Glick*, 1949).

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<sup>1</sup>) This work was aided by a grant from Statens Medicinska Forskningsråd (The Medical Research Council of Sweden).

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*Hench et al.* (1949) showed that adrenocorticotrophic hormone (ACTH) has a dramatic clinical effect in rheumatoid arthritis. The specific mechanism of ACTH in this disease is unknown. It is conceivable that there may be a common factor in the variations of the hyaluronidase inhibitor observed in some of the above-mentioned pathological states and the clinical effect of ACTH.

In order to gain some information relative to this point, the present investigation was concerned with the variations that occur in the level of the hyaluronidase inhibitor in the blood serum of a patient with rheumatoid arthritis treated with ACTH protein and ACTH peptide.

## MATERIAL AND METHODS

*Case report.* The patient was a 58 year old man suffering from severe rheumatoid arthritis. The clinical findings will be given extensively in a later publication. The patient was treated between November 6 and 10 for four days, see Fig. 1) with a daily dose of 25 mg. ACTH protein. This was followed by a control period of eight days. He was then treated for another four days (between November 18 and 22) with 13 mg. of ACTH peptides daily. All doses were divided into six daily injections.

The ACTH protein and ACTH peptide used in this study were kindly at our disposal by dr. C. H. Li. The ACTH was isolated from sheep pituitary glands by the method described by Li *et al.* (1942-43), and the ACTH peptide preparation was obtained by pepsin digestion as described by Li (1948).

The determination of the hyaluronidase inhibiting property of serum (Glick & Gollan, 1948, Hakanson & Glick, 1949). The hyaluronidase was prepared from bovine testicle and the hyaluronic acid from human umbilical cord. Hyaluronidase inhibition was expressed as the per cent inhibition effected by 0.02 ml. of blood serum under the conditions employed. The per cent inhibition was defined as  $\frac{(100 R - R_0)}{R}$  where  $(R_0)$  is the time required to reduce the viscosity of the reaction mixture to one-half its original value, and  $(R)$  is the time required to reduce the initial viscosity to one-half after incubation of the 0.5 ml. of enzyme solution with 0.02 ml. of serum and 1.48 ml. of water for 10 minutes at 37.5° C. Because the inhibitor loses activity rapidly on standing at room temperature, it is im-

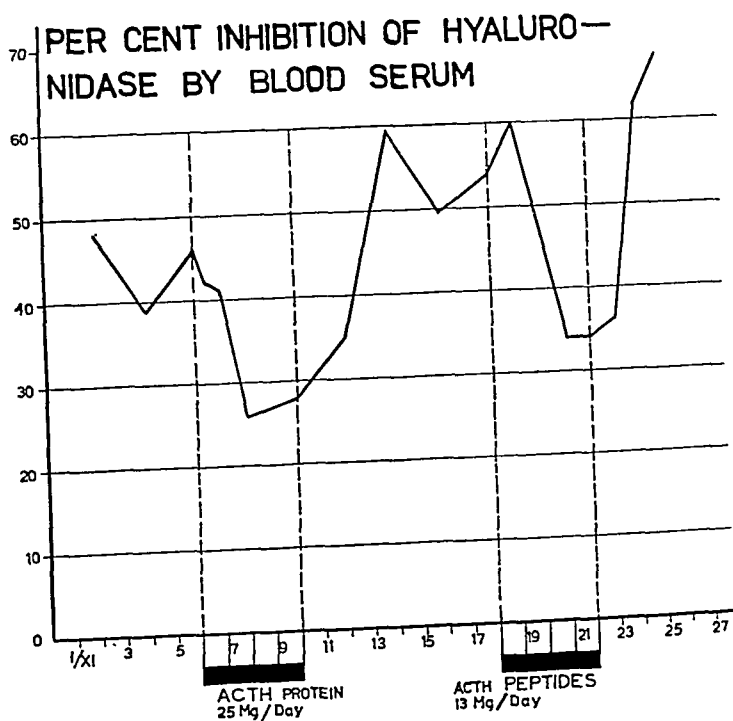
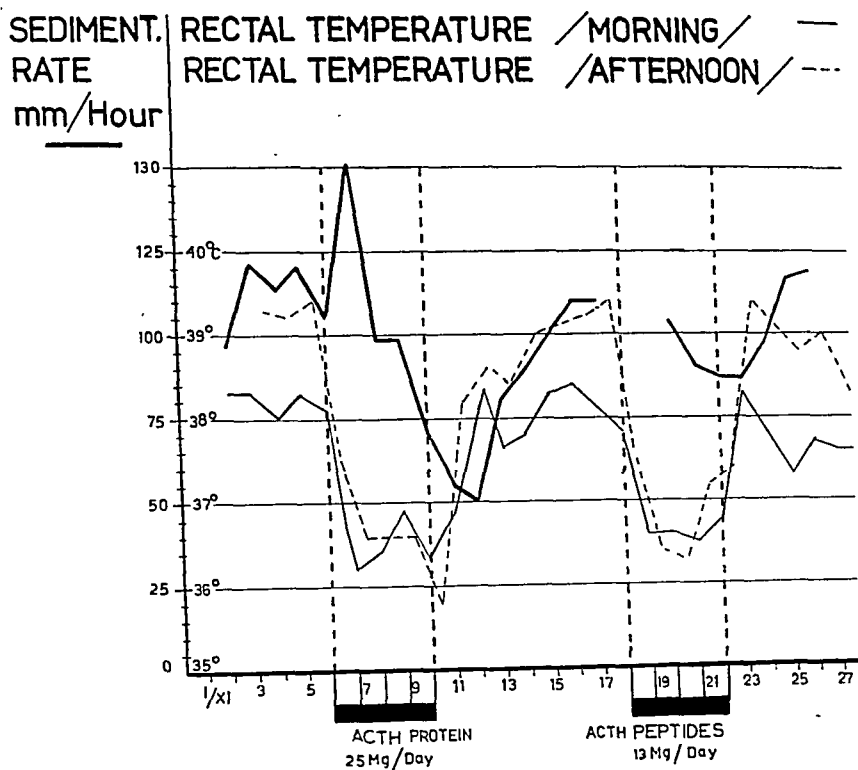


Fig. 1.

The effect of ACTH administration on sedimentation rate, rectal temperature and hyaluronidase inhibition of blood serum in the present case.

portant that the collection of samples be as uniform as possible. The blood was collected in clean tubes, allowed to clot, centrifuged, and the serum removed and immediately frozen. To eliminate differences in various batches of enzyme and substrate, all serum samples were stored at  $-20^{\circ}$  C. until the collections were complete. All of the samples were then assayed during the same 24 hour period using the same enzyme and substrate preparations.

## RESULTS

The improvement during treatment was a most dramatic one appearing three to four hours after the first injection of 4 mg. of ACTH protein and 2 mg. of ACTH peptide. After withdrawal of the hormone the patient's symptoms returned within 6—8 hours.

Fig. 1 shows the changes of rectal temperature and sedimentation rate. While the fall of temperature was an immediate one, the fall of sedimentation rate was not evident for the first 24 hours of treatment. After termination of treatment the same lag of reaction of the sedimentation rate was noted.

From Fig. 1 it is obvious that there was a significant fall of the hyaluronidase inhibitor values during administration of ACTH protein (25 mg. per day) and ACTH peptide (13 mg. per day) for four days. The fall in hyaluronidase inhibitor did not correspond with the clinical effect — including body temperature — in that the clinical effect was immediate, while the inhibitor level did not markedly change for a period of 24 hours after the first injection of ACTH protein or peptide. This lag was also observable after termination of treatment. The symptoms reappeared within 6—8 hours, while the inhibitor did not regain its pretreatment level for 24—48 hours.

There seems to be some time correlation between the variations of the sedimentation rate and the inhibitor level.

These preliminary results seem to indicate that the lowering of the hyaluronidase inhibitor level during administration of ACTH protein and peptide may be secondary to the effect of the hormone on the patient's clinical condition in-

cluding for example the lowering of the body temperature. Further investigations are in progress in the hope of shedding more light on this point.

### SUMMARY

In a patient with rheumatoid arthritis treated with ACTH protein and ACTH peptide, the hyaluronidase inhibitor was found to decrease during treatment and to increase to pre-treatment levels after withdrawal of the hormone.

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THE EFFECT OF ADRENOCORTICOTROPHIC  
HORMONE (ACTH) AND ADRENOCORTICO-  
TROPICALLY ACTIVE PEPTIDES (ACTH PEP-  
TIDES) ON THE CIRCULATING EOSINOPHILS  
AND URINARY EXCRETION OF ADRENALINE  
AND NOR-ADRENALINE IN A HUMAN  
SUBJECT<sup>1)</sup>

BY

U. S. VON EULER and ROLF LUFT

Early in this century *Babes & Jonesco* (1908) were able to demonstrate an enlargement of the adrenals in rabbits after repeated administration of adrenaline intravenously. Later investigations by *Vogt* (1945) and *Long* and his group (1945, 1947) showed the stimulating effect of adrenaline on the adrenal cortical function. This stimulation was prevented by hypophysectomy.

Further *Bertelli et al.* in 1910 noted eosinopenia in dogs after intravenous administration of adrenaline. These studies were extended to human subjects by *Hills & Thorn* (1948), *Thorn & Forsham* (1949), and *Hortling & Pekkarinen* (1949) who showed that adrenaline intravenously induced a fall in circulating eosinophils in the presence of an intact pituitary-

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<sup>1)</sup> This work was aided by a grant from Statens Medicinska Forskningsråd (The Medical Research Council of Sweden).

adrenal system. It is now well established that ACTH causes in human subjects a fall in circulating eosinophils and lymphocytes, when the adrenal cortex is intact.

These findings indicate some kind of interrelationship between adrenaline, one of the hormones of the suprarenal medulla, the release of ACTH from the pituitary gland, and adrenal cortical function.

During the last two years much interest has been given to nor-adrenaline (amino-ethanolcatechol) or non-methylated adrenaline. *Euler* (1946) has shown that this substance is the predominant neuroergone in adrenergic nerves, and its rôle as mediator substance is now well established. Investigations by *Luft et al.* (1950) in healthy human subjects showed that intravenous administration of nor-adrenaline did not induce the eosinopenia and lymphopenia elicited by adrenaline.

It was the aim of the present investigation to study the effect of ACTH protein and ACTH peptides (*Li*, 1948) on the circulating eosinophils and the excretion of catechols (adrenaline and nor-adrenaline) in the urine in a human subject.

## MATERIAL AND METHODS

*Case report.* The studies were performed on a 58 year old man suffering from a severe rheumatoid arthritis. The clinical findings will be given extensively in a later publication. The patient was treated for four days (between November 6 and 10) with a daily dose of 25 mg. of ACTH protein. This period was followed by a control period of eight days. He was then treated for another four days (between November 18 and 22) with 13 mg. of ACTH peptides daily. All doses were divided into six daily injections.

*The ACTH protein and ACTH-peptides* used in this study were kindly put at our disposal by dr. C. H. *Li*. The ACTH was isolated from sheep pituitary glands by the method described by *Li et al.* (1942, 1943), and the ACTH peptide pre-

paration was obtained by pepsin digestion as described by *Li* (1948).

*The determination of catechols in urine.* The catechols in urine were determined according to the following procedure. The total 24-hour amount of urine was collected and stored in the refrigerator with hydrochloric acid added to give pH 3.5—4. A sample of 300 ml. was hydrolysed with sulphuric acid at pH 2.5—3 for 20 minutes at 100° C. The pH was checked repeatedly during the hydrolysis. By this treatment conjugated catechols were split as shown by *Richter* (1940).

To the hydrolysed urine was added 1.5 ml. 20 p.c. aluminium sulphate per 100 ml. urine and aluminium hydroxide precipitated by addition of 0.5 n NaOH to pH 7.6 during continuous stirring. The precipitate was filtered off, washed twice with distilled water, and dissolved in n H<sub>2</sub>SO<sub>4</sub>. After adjustment of pH to 3.5 with 0.5 n NaOH 4 volumes of a mixture of equal parts of ethanol and acetone were added and the precipitated salts filtered off after some hours' standing in the cold. The filtrate was freed from ethanol and acetone by evaporation in vacuo and taken down to a small volume. The extract thus obtained could be used for biological assay on the cat's blood pressure and the hen's rectal caecum as described earlier (*Euler*, 1949).

The determination of *eosinophil leucocytes* were done with a method described by *Rud* (1948).

## RESULTS

### *Clinical Findings.*

An extensive report of the clinical findings will be given in a later paper. The improvement was most dramatic and appeared already three to four hours after the first injection of 4 mg. of ACTH protein and 2 mg. of ACTH peptides.

### *Circulating eosinophils* (Fig. 1).

These showed a rapid and marked decrease. Twentyfour hours after the first injection of ACTH peptides the eosino-



phils had dropped to zero. They returned to the original value very rapidly after the hormone was withdrawn. The return was prolonged after 25 mg. of ACTH protein daily in contrast to the return of the eosinophilic count after 13 mg. of ACTH

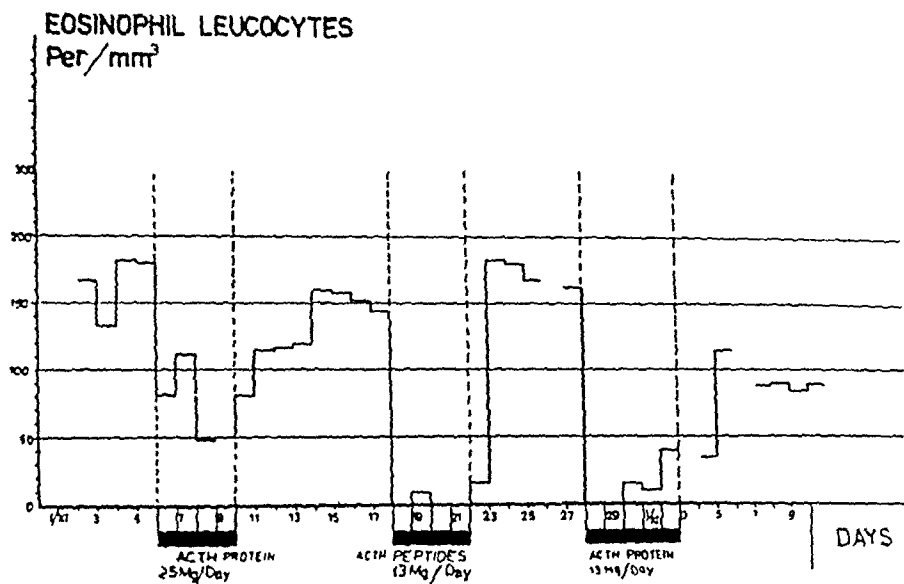


Fig. 1.

The effect of ACTH administration on the number of eosinophil leucocytes in the present case.

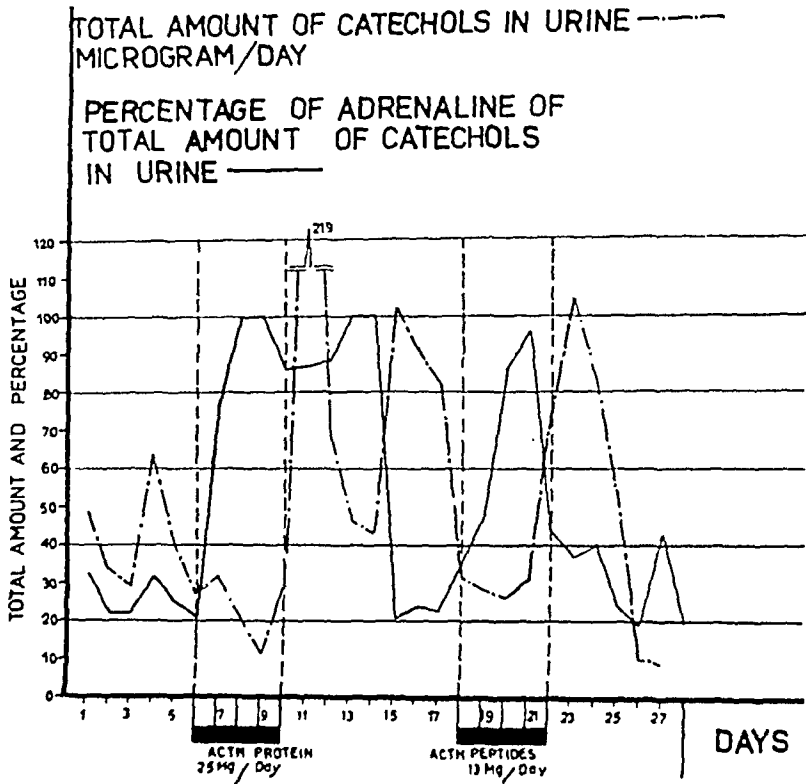
peptides daily where the pretreatment values were reached in 24 hours.

*The excretion of total amount of catechols (Fig. 2).*

The excretion of the total amount of catechols showed fluctuations that to a considerable extent followed the injection periods. During the days of injection the total catechols in urine showed some of the lowest values obtained. The day after the last injection of ACTH protein the excretion showed a peak that was most prominent, wherafter the excretion returned to the pretreatment level. The excretion then increased again. During the administration of ACTH peptides another low level of catechols in urine was reached and another peak of excretion followed the withdrawal of the peptides.

*The percentage of adrenaline of total amount of catechols in urine.*

The adrenaline percentage curve had a somewhat similar shape as the excretion curve of the total amount of catechols



*Fig. 2.*

The effect of ACTH administration on the amount of catechols in urine and on the percentage of adrenaline of total amount of catechols in urine in the present case.

but appeared already at the beginning of the injection period. During the injection periods the percentage of adrenaline excreted increased significantly. The period of increase was prolonged during and after the daily administration of 25 mg. of ACTH protein compared to 13 mg. of ACTH peptides daily.

## CONCLUSIONS

The present study on a limited scale has shown:

that administration of ACTH protein and ACTH peptides are paralleled by a low excretion of total catechols in the urine and that a highly increased excretion follows immediately the withdrawal of ACTH;

that during the administration of the hormones mentioned the percentage of adrenaline of the total amount of catechols excreted increased significantly.

The limited amount of experimental data makes any extensive discussion impossible. However, the relatively low excretion of catechols during the administration of ACTH protein and peptides, and the high peak of excretion after withdrawal of the hormones suggests an antagonistic effect of ACTH on the formation or release of catechols in the body. Since the catechols — at least adrenaline — have been shown to stimulate the release of ACTH from the pituitary, and since the present investigation indicates that ACTH antagonizes the release of catechols, this would mean that there exists not only a stimulating effect of catechols on the release of ACTH from the pituitary gland, but also an inhibiting action of ACTH on the release of catechols — presumably mainly in the adrenal medulla.

The percentage of the adrenaline of the total amount of catechols increases during ACTH administration but the significance of this finding is not clear. It is in parallel with other findings in patients during ACTH administration, for instance the reduction of circulating eosinophils as shown in Fig. 1. Further studies are in progress dealing with the interrelationship between ACTH protein and peptides and the catechol group.

## SUMMARY

ACTH protein and ACTH peptides (*Li*, 1948), 25 mg. and 13 mg. respectively administered daily for four days to a 58 years old man with rheumatoid arthritis, caused a lowered excretion of the total amount of catechols (adrenaline and nor-adrenaline) in the urine during the injection period. The withdrawal of ACTH was immediately followed by a highly increased excretion of catechols for some days.

The percentage of adrenaline of the total amount of catechols in the urine and the excretion curve for total catechols showed some similarity but were shifted in phase to each other. Thus, the percentage of adrenaline increased during the injection periods and decreased during the control periods.

The eosinophils showed a rapid and marked decrease after ACTH protein as well as ACTH peptides.

The technical assistance of miss Sigbrit Hellner is gratefully acknowledged.

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## DETERMINATION OF THYROTROPHIN BY MEANS OF RADIOACTIVE PHOSPHORUS

BY

ULF BORELL and HJALMAR HOLMGREN

### HISTORY

Up to the present the thyrotrophin content of a preparation has been mainly determined by two different methods, viz. either by determining the direct effect of the hormone on the thyroid gland or by studying changes brought about by the increased secretion of thyroid hormone. At the third international conference on standardisation of hormones in Geneva, 1938, where directions for the standardisation of different hormones were given, it was agreed that only the method of determining the direct effect on the thyroid was to be used.

Injections of thyrotrophin produce an increase in the weight of the thyroid. *Rowlands & Parkes* (1934) and *Andersen* (1943) utilized this increase as a means of determining the hormone quantitatively, defining the unit as the amount of hormone that would double the weight of the thyroid in guinea pigs. In order to make the method more accurate *Smelser* (1938) suggested the use of new-born chickens. *Kabac & Liapin* (1938) and *Cope* (1938) showed that these animals were four times as sensitive as guinea pigs. Subsequent investigations, however, have shown that chickens of different sex react differently (*Bergman & Turner*,

1939), as do chickens from different breeders (*Bates, Riddle & Lahr, 1941*).

The changes in the histological picture of the thyroid, produced by thyrotrophin, have also been studied in order to obtain a measure of the thyrotrophin content of different preparations. The height of the follicle cells has in particular been used (*Junkmann & Schoeller, 1932*). They defined the unit for guinea pigs as the smallest daily dose, which given for three days, would produce distinct changes in the histological picture. In order to make this method more objective *Heyl & Laqueur (1934)* suggested that the picture be compared with a series of standard pictures showing increased thyroid activity.

*Rawson & Salter's* method (1940) was a great improvement. Instead of a subjective evaluation of the height of the follicular cells they suggested the use of an ocular micrometer for measuring the height. In the thyroid of a chicken a distinct cell was measured in each of 100 follicles. As little as one eighth of a Junkmann-Schoeller unit gave a measurable difference in height. With this method, however, the determinations were restricted to a relatively small range of dosage.

Recently *Lever (1948)* has published the principles of a new mathematical method for the determination of the state of activity of the thyroid gland. This method is based on the relations between cellnumber, the height of the follicle cells and the outer and inner diameter.

*Stimmel, McCullagh & Picha (1936)* followed entirely different lines in testing preparations. They demonstrated that the iodine content in the thyroid gradually decreased after injections of thyrotrophin.

*Borell (1945)*, in a comprehensive study demonstrated that repeated administration of very small doses of thyrotrophin to guinea pigs caused a rapid increase in the cell height. He also observed a simultaneous and parallel change in the phosphate content. Thus, by estimating the phosphate content, a direct measure of the increase in the cell height was obtained.

The high follicular cells probably have a high metabolic

rate. As in other cells in the body, energy is in all probability produced by the rapid metabolism of carbohydrate and of the phosphorylated intermediary products. Therefore, if it were possible to determine not only the content of phosphate but also the rate at which this substance is taken up and converted in the thyroid, we should have a method that would be accurate and that would also allow of titration within a wider range of dosage. This possibility has now been realised, following the introduction of radioactive phosphorus.

### MATERIAL AND METHODS

In the present experiments 87 male guinea pigs, weighing about 150 gm., were used. The advantage of using young animals is that their thyroids are relatively inactive, with little variability among different animals. In order to get the histological picture of the different thyroids still more uniform, the guinea pigs were kept at a temperature of  $+30^{\circ}$ — $32^{\circ}$  C, as the animals' metabolic rate is then at its lowest. At this temperature the foci of increased activity which may be found now and then in the inactive glands of young animals are entirely eliminated. Another advantage is that the variations in activity due to seasonal changes in temperature are avoided. After full fortnight in this temperature, the animals were given daily injections of very small amounts of thyrotrophin (Aminon, Pharmacia) for two days. They were killed by a blow on the back of the head 24 hours after the last injection. According to *Borell* (1945) the most marked increase in the phosphate content is observed after the injection of relatively small quantities of the hormone daily for two days. Therefore, we considered it best to choose this period, as it would probably provide the most suitable conditions for accurate assay, and would also allow of measurements within a wider range of dosage. The amount of hormone given with each injection varied between  $\frac{1}{2}$  and 12 guinea-pig units (GPU) assayed by the method of *Heyl & Laqueur*. The volume of each injection was 1 ml.



Radioactive phosphorus  $P^{32}$  in a dose of 0.05 mC dissolved in 5 per cent glucose was injected intraperitoneally into all the animals 40 minutes before they were killed. The thyroid lobes were weighed and the left lobe was then analysed for  $P^{32}$  content and total phosphate. In order to obtain quantities sufficiently large to determine the phosphate as well as the radioactivity, it was necessary to use the thyroid lobes from three animals.

The amount of phosphate was determined colorimetrically (according to *Brigg's* method, 1922). The radioactivity was measured with a Geiger-Müller counter in which  $\beta$ -rays from disintegrating phosphate atoms were intercepted (*Levi*, 1941. *Lindberg*, 1946). The impulse was enlarged and registered on a mechanical counter. On the whole, the number of impulses should thus be proportional to the number of disintegrating phosphate atoms.

The radioactivity is as a rule expressed as the number of impulses discharged by the preparation per minute. The values are also expressed as specific activity, which means that the number of impulses per  $\gamma$  P has been calculated (specific activity =  $\frac{\text{number of impulses}}{\gamma \text{ P}}$  ).

The right lobe was used for the histological examination and for measuring the cell height. The number of follicle cells measured by means of the ocular micrometer in each thyroid was 25. This gave mean values with only relatively small standard errors. *Griesbach & Purves* (1943) have shown that representative values may be obtained by measuring 20 cells.

## RESULTS

The results of some of our experiments are recorded in Table 1 and Fig. 1. In these experiments we used a thyrotrophin preparation, the hormone content of which has not yet been determined, but in which the amount was presumed to be equivalent to 200 guinea-pig units (GPU) per ml. The

figures represent the mean of three experimental series. Different amounts of thyrotrophin were injected into the guinea pigs, and three animals were used for each dose in the respective series. Thus, each value in the table represents on an average the examinations of nine thyroid glands. In order to obtain comparable values in the different experiments, the radioactivity and phosphate content of the thyroids of the controls were taken as 100 and the other values were calculated in relation to this. The cell height was calculated in the same way.

TABLE 1

Guinea pigs treated with thyrotrophin (Ambinon, preparation 1) and  $P^{32}$ . Radioactivity and height of follicle cells of the thyroid. Value for the controls = 100.

Nr. of GPU *) thyrotrophin	$P^{32}$	$P^{32}/\gamma P$ (Spec. activity)	Height of follicle cells
—	100	100	100
$\frac{1}{2} \times 2$	94	108	109
$1 \times 2$	134	204	110
$2 \times 2$	180	224	108
$4 \times 2$	243	301	115
$6 \times 2$	280	369	128
$8 \times 2$	372	381	136

\*) Guinea Pig Unit.

The number of impulses  $P^{32}$  in the thyroid was found to increase progressively with increasing doses of thyrotrophin. The highest number of impulses was observed after the injection of 16 GPU of thyrotrophin. The changes in specific activity were parallel to those in the number of impulses. This shows that the phosphate content had not changed. With regard to the cell height, a very small progressive increase was observed after a total of 8 GPU of thyrotrophin had been injected. In some of our earlier experiments corresponding amounts produced a distinct increase in the cell height. The failure to obtain an increase in the present experiments was probably due to the fact that the preparation did not contain the assumed amount of thyrotrophin.

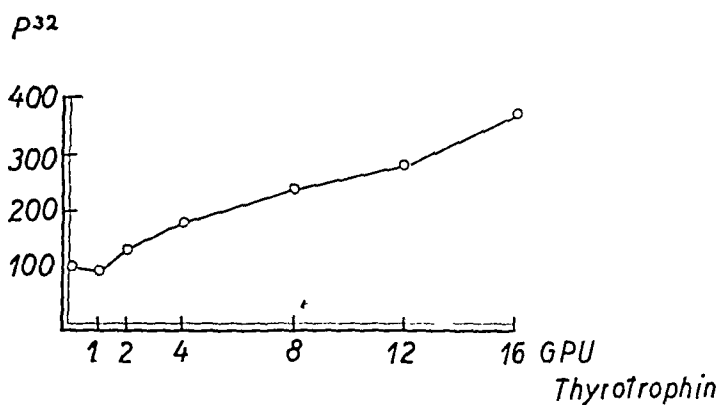
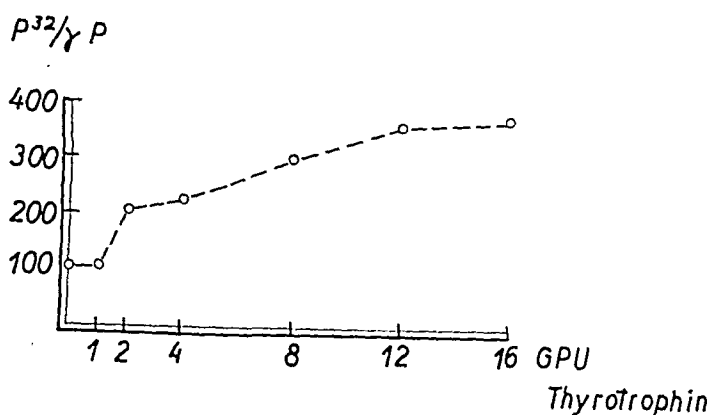
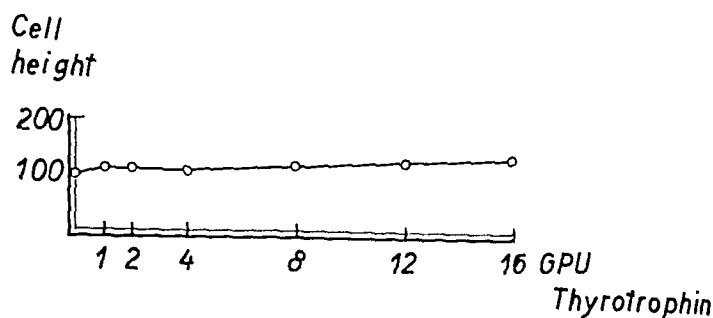


Fig. 1.

Changes in the height of the follicle cells, specific radioactivity and  $P^{32}$  content of the thyroid after injection of different amounts of thyrotrophin (Ambinon, preparation 1). The values are expressed as a percentage of the normal value.

However, the results show that there is a marked progressive increase in the amount of radioactivity in the organ, in spite of the fact that the amount of hormone is not large enough to produce distinct changes in the cell height. The experiments also show that it is only necessary to determine the number of impulses. It is not necessary to calculate the specific activity, and analysis of phosphate can thus be omitted. For all the examinations, the thyroid of one guinea pig is all that is required. The one lobe is used for the analysis of radioactivity, the other for the histological examination and the determination of the cell height.

TABLE 2

Guinea pigs treated with thyrotrophin (Ambinon, preparation 2) and  $P^{32}$ . Radioactivity and height of follicle cells of thyroid.

Nr. of GPU thyrotrophin	$P^{32}$ . Number impulses per minute in 1 thyroid lobe	$P^{32}$ in % Value for controls = 100	Height of follicle cells Value for controls = 100
—	22.2	100	100
$\frac{1}{2} \times 2$	31.8	143	100
$1 \times 2$	37.8	170	113
$2 \times 2$	118.5	535	130
$4 \times 2$	136.5	615	143
$6 \times 2$	217.0	978	151
$8 \times 2$	237.0	1070	163
$12 \times 2$	164.0	740	160

Table 2 and Fig. 2 show the results of an experiment in which this technique was used. Each point on the curve represents the mean of three values. On this occasion an Ambinon preparation with a content of 200 GPU per ml., determined according to *Heyl & Laqueur*, was injected into the guinea pig.

A progressive increase in the cell height as well as in the amount of radioactivity was observed. The number of phosphate atoms recorded was multiplied more than fivefold after the injection of 4 GPU of thyrotrophin. The cell height also increased considerably. The highest values for the cell height as well as for activity were obtained after injections of, all-

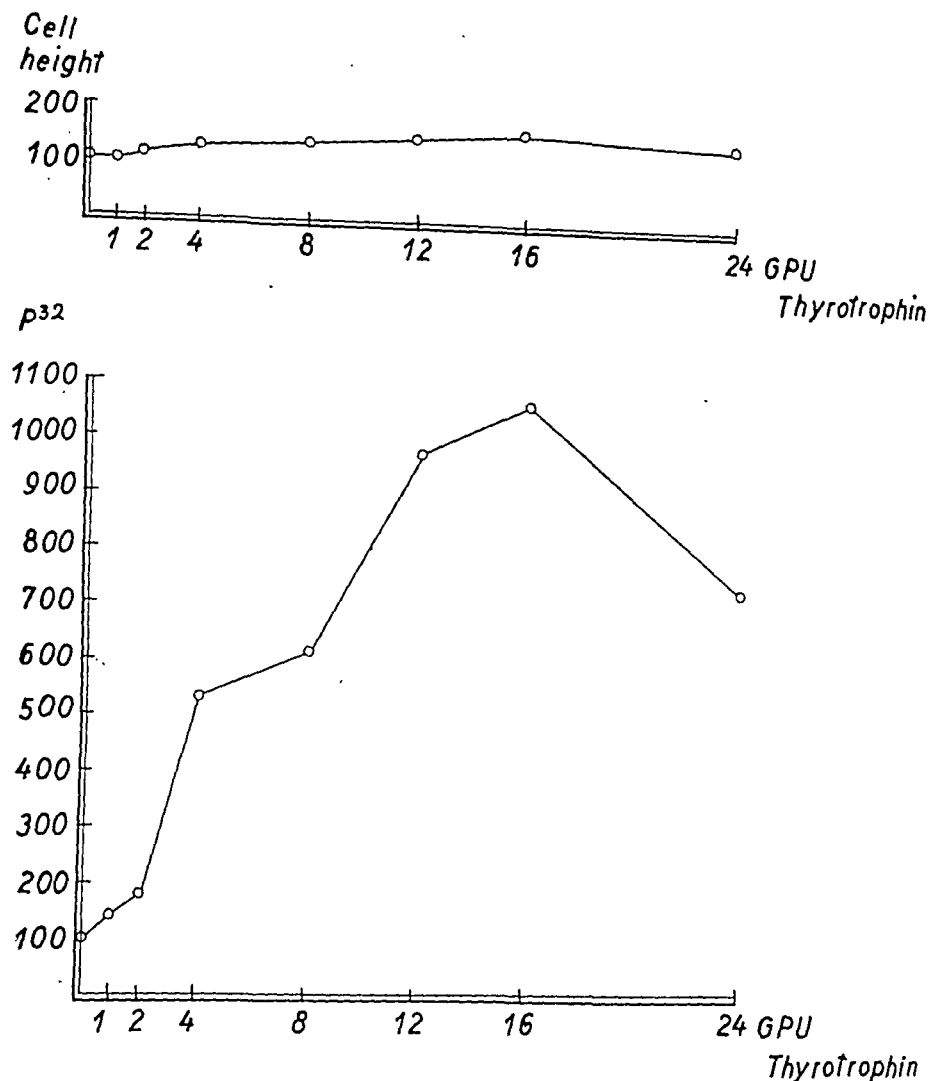


Fig. 2.

Changes in the height of the follicle cells and  $P^{32}$  content of the thyroid after injection of different amounts of thyrotrophin (Aminon, preparation 2). The values are expressed as a percentage of the normal value.

together, 16 GPU of thyrotrophin. The number of impulses in the thyroid lobe was multiplied more than tenfold, while the cell height was hardly doubled. No change in the fresh weight was observed.

## DISCUSSION

Many factors must be considered in the working out of a method for the standardisation of a hormone. The method should be accurate, and this is of particular importance in the determination of the hormone content in organs and body fluids. It should, preferably, be suitable for a relatively wide range of dosage. Further, its application should not be complicated or time-consuming.

At present our most accurate method for the assay of thyrotrophin is that of measuring the height of the follicular cells (see *Borell*, 1945). A definite increase in the number of radioactive phosphate atoms in the thyroid, however, may be observed after the injection of thyrotrophin in quantities which produce only a slight increase in the cell height. Injections of  $P^{32}$  make titration possible within a relatively large range of dosage. The fact that the reactions are very marked also seems to be of great advantage. Whereas the cell height after suitable hormone treatment is hardly doubled, the amount of radioactivity is multiplied at least tenfold. The measuring of the cell height by means of an ocular micrometer will always involve a subjective factor. Different examiners choose different cells. The fact that the cell borders facing the follicular lumen and the interfollicular tissue may be ill-defined makes the values uncertain. True, these factors may be partly eliminated by measuring a larger number of cells. This, is however, time consuming unlike the method of determination by radioactivity. The latter method is moreover completely objective.

In the present experiments we used different preparations of thyrotrophin. One of them was found to contain barely one third of the expected content, as was evident from the measurement of both the cell height and radioactivity.

In a previous experiment it was demonstrated that the cell height increased parallel to the phosphate content of the gland. In a normal thyroid a cell height of  $8.3 \mu$  was recorded, while the phosphate content amounted to  $22.5 \gamma$ . Injection of 2 GPU of thyrotrophin daily for three days gave a cell height of

12.9  $\mu$  and a phosphate content of 32.3  $\gamma$ , i. e., an increase of 55 per cent and 44 per cent, respectively (Borell, 1945). In the present experiments the injection of 6 GPU of thyrotrophin daily for two days produced an increase in the cell height of 51 per cent, whereas the amount of radioactive phosphorus was increased by no less than 878 per cent.

The growth of the follicle cell requires the administration of phosphorus, which accounts for the parallel increase in the cell height and the amount of phosphorus. The enormously increased uptake of radioactive phosphorus isotope after injections of hormone may have several explanations. In the hormone-stimulated thyroid a very lively exchange of phosphorus must take place between the follicular cells and the blood stream. The reason for this may be that the permeability of the follicular cells is greater after injections of thyrotrophin. It is also possible that a much more rapid metabolism of phosphorylated carbohydrate esters takes place in the cells of the gland after injections of thyrotrophin and that the number of labelled atoms are thus increased in the gland. On the basis of the present experiments it is not possible to determine the true cause for the marked increase of  $P^{32}$  in the activated gland. The reason is probably to be found in an increase in the cell height, in the permeability and in the metabolism of phosphorylated carbohydrate esters.

### SUMMARY

The possibilities of using radioactive phosphorus ( $P^{32}$ ) in the determination of the amount of thyrotrophin has been investigated. The following observations were made:

1. The amount of radioactive phosphorus in the thyroid gland increases markedly after injections of thyrotrophin.

After effective stimulation with thyrotrophin, the number of labelled phosphate atoms in the thyroid is multiplied nearly tenfold, whereas the cell height is barely doubled.

2. A distinct increase is produced even by small amounts of thyrotrophin. Moreover, the method is quite as accurate as that which depends on the measurement of cell height.
3. The radioactive phosphorus in the thyroid progressively increases after injections of thyrotrophin within a wide range of dosage.
4. The method used seems to be valuable in the assay of thyrotrophin. It is objective and easily applied.

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GYNECOMASTIA, HYPERTENSION, DECREASED  
DEXTROSE AND INCREASED INSULIN TOLERANCE  
IN A CASE WITH DIFFUSE BILATERAL ADRENAL  
CORTICAL HYPERPLASIA, ADRENAL CORTICAL  
ADENOMA, AND PITUITARY CHANGES\*)

BY

ROLF LUFT and BJÖRN SJÖGREN

The term Gynecomastia is used to denote an enlargement of the breast in the male due to hyperplasia of the duct epithelium and periductal stroma. It may be produced by an altered hormone secretion in the testicles and adrenal cortex or a disordered metabolism of these hormones. It is also probable that the end organ might show an increased sensitivity to the hormones mentioned. This latter mechanism has, however, not so far been elucidated.

Gynecomastia has thus been found in cases with tumours (*Hunt & Budd, 1939*) or degenerative changes of the testicles (*Klinefelter et al., 1942, Heller & Nelson, 1945*) with or without an increased excretion of oestrogenic or gonadotrophic hormones; in tumours of the adrenal cortex with or without an increased excretion of oestrogens (*Bittorf, 1919, Holl, 1930, Broster & Vines, 1933, Lisser, 1936, Simpson & Joll, 1938, Roholm & Teilmann, 1942, Mc Fadzean, 1946, Armstrong & Simpson, 1948, Broster & Patterson, 1948, Wilkins, 1948*), in nu-

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\*) This case was kindly referred to us by professor Nils Antoni, Serafimerlasarettet.

tritional disturbances with or without changes in the hormone excretion (*Salter et al.*, 1947, *Klatskin et al.*, 1947). and in liver diseases with an increased excretion of oestrogens (*Edmondson et al.*, 1939). The significance of the pituitary gland in the development of gynecomastia has not been elucidated. but gynecomastia has been described in cases with pituitary tumours (*Goodman*, 1937).

In the present case of gynecomastia hypertension and an altered carbohydrate metabolism occurred at the same time. The pathological-anatomical findings included a diffuse bilateral adrenal cortical hyperplasia, a large adrenal cortical adenoma, and cytological changes in the anterior lobe of the pituitary gland.

### CASE HISTORY

*Past history.* T. L., captain, born 1888. Admitted to the endocrine department in November 1947.

Married and had three healthy children. Operated on in 1928 for gastric ulcer; in 1930 cholecystectomy. Had malaria in 1932, and was operated on in 1945 for papilloma of the bladder. Admitted to the neurological department of Serafimerlasarettet because of pains in the neck and left arm and paraesthesias of the hypothenar region for the last one and a half years. The diagnosis of polyneuritis was made. For the last four months he had noticed an increasing swelling and tenderness of both breasts. There was no history of trauma to the breast.

*Physical examination.* A very thin man of medium height with diffuse dark pigmentation of the skin. No pigmentation of the mucous membranes. Hairiness of a masculine type, voice deep. Penis, scrotum, testicles and prostate gland of normal size. The breasts were rounded with an increased amount of fat. The glandular tissue in each breast was of the size of a walnut, tender on palpation.

No signs of cardiac failure. Blood pressure 180/110 mm. Hg. Examination of the eyes disclosed hypertonic retinopathy. The liver was palpable at the costal margin, smooth and slightly firm but not tender. The lower pole of the left kidney was palpable. Neurological examination showed impaired muscular power of the left hand and numbness of the left hypothenar region.

X-ray examinations of the skull, cervical spine, heart and lungs

showed normal findings. Encephalography and myelography normal. Urography and insufflation of oxygen around the left kidney disclosed a large tumour localized above the kidney and islocating it downwards.

*Laboratory findings.* Red blood cells 3.5 mill., hemoglobin 70 per cent, white cells 6000, diff. count normal. Urine findings normal. NPN 26 mg. per cent. In serum: total proteins 6.7 per cent, iron 122 ; per cent, chlorides 336 and cholesterol 210 mg. per cent, thymol turbidity test 0.7 units, Takata-Ara negative, galactose tolerance test with 0.9 gm. excreted in 4 hours. Kepler's test gave an index of 48. Sternal bone marrow normal. Glomerular filtration rate (creatinine) 110 ml./min.

Dextrose tolerance test (two-dose, one-hour, capillary blood) 109—213—291 mg. per cent, 0.6 gm. dextrose excreted. Insulin tolerance test (6 I. U. intravenously, body weight 58 kg.) without any hypoglycemic symptoms during the test, fasting blood sugar value 105 and lowest value 75 mg. per cent.

*Hormone excretion.* Gonadotrophin less than 20 M. U. per day. Oestrogens between 250 and 500 I. U. per day. 17-ketosteroids 6 mg. per day.

The patient was operated on in December 1947 (Thorsén). A tumour the size of a grape-fruit was found above the left kidney but completely separated from it. The left adrenal was markedly enlarged and divided in several pieces, attached to the capsule of the tumour. The latter was extirpated. The patient died two days later with symptoms resembling those of acute shock.

*Findings at autopsy:* The heart weighed 360 gm. and was of normal size and shape. Myocardium normal. Slight lipoidosis in the coronary arteries. Moderate atherosclerosis of the aorta. Lungs with atelectasis of the lower lobes. The pleural cavities contained 600 and 700 ml. of transudate respectively. More than 1000 ml. of blood and blood clots were found in the small pelvis, around the spleen and at the site of operation. The spleen weighed 120 gm. and was anemic. The stomach rest (resection 1928), intestines, mesentery, liver, pancreas, brain and meninges normal on macroscopic examination.

*The thyroid* was of normal size and had a normal cut surface. *Parathyroids* normal. No thymic rests. The right adrenal was enlarged, weighed 13 gm., and had a broadened cortex rich in lipoids. On cross section through the middle, the folded cortex measured about 5—7 mm. in thickness. No medulla could be seen. No adrenal adenomas were found. The left adrenal had a central part and a number of parts scattered on the tumour capsule. They could all be easily removed from the tumour capsule, and seemed to be

completely free from it. The total weight of the left adrenal was 20 gm. It contained exclusively cortical tissue rich in lipoids. The *testicles*, prostate gland, and seminal vesicles looked normal. The hypophysis was macroscopically normal.

*Microscopic examinations.*<sup>1)</sup> The hypophysis (stained with hematoxylin-eosin and according to Ladewig): Posterior lobe normal. A large colloid cyst was found at the border of the anterior lobe. No

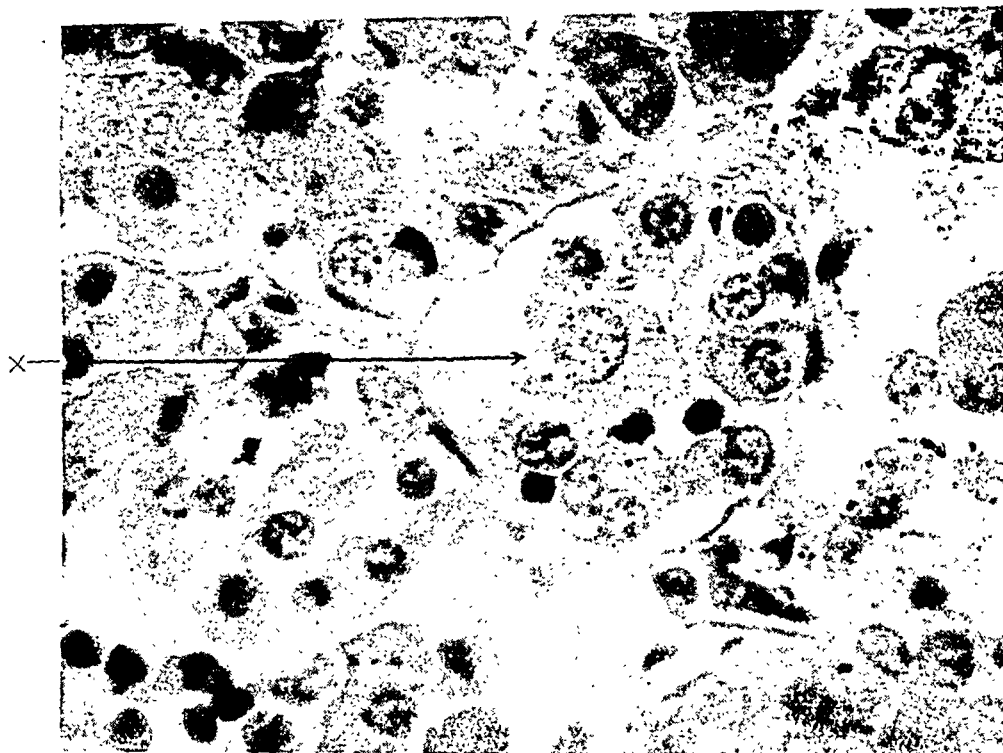


Fig. 1.

The anterior lobe of the hypophysis. At X hypertrophic amphophil. — Ladewig. —  $\times 400$ .

adenomas. In the anterior lobe the percentage of acidophils was normal, a number of them showing a sparse granulation. The chromophobes were mostly larger than normal. A fairly large number of those cell types were seen, which were first described by *Mellgren* (1945) in virilism. They were hypertrophic, had ill defined cell margine and a foamy amphophilic cytoplasm. Their nuclei

<sup>1)</sup> We wish to express our thanks to Professor Olle Reuterwall, Professor Jan Mellgren, Docent Kaj Lindberg, and Dr. Bertil Falconer for their most valuable advice about the patho-anatomical findings.

were large, polymorphous and poor in chromatin (Fig. 1). No hyaline basophils were seen.

The adrenal cortex was considerably enlarged, the enlargement involving mainly the zona fasciculata. The zona glomerulosa had a normal width, while the zona reticularis was somewhat widened. The cells of all three cortical layers contained an abundance of lipoids (Sudan III and Scharlach R), which were anisotropic. The Ponceau-fuchsin staining gave a negative result, and no marked pigmentation of other kinds was found.

The adrenal tumour was completely surrounded by a fibrous capsule. The cut surface was yellowish and in parts yellow-red due to several small hemorrhagic and necrotic parts. The material was fixed in formalin and embedded in paraffin. In the sections the tumour cells were arranged in cord- and bundle-like solid strands mainly divided by thin connective tissue septa often rich in dilated capillary vessels. The cells were of a cortical type with a definite and rather abundant amount of clear or finely granular cytoplasm. Some cells showed degenerating cytoplasm, and had high contents of lipid droplets (Sudan III and Scharlach R). The nuclei were sometimes small and dense, but in most cases fairly large, and of rounded rather symmetrical shape with scanty chromatin contents, distinct margins and generally with one, and sometimes 2—3 nucleoli of varying size. No obvious cellular or nuclear polymorphism and no cells in mitosis could be observed. Rather typical were the cells next to the connective tissue septa which were of a somewhat cylindrical shape and arranged perpendicularly to the septa, with the nuclei asymmetrically located at the cell poles opposite the septa.

The tumour capsule was formed by a moderately thick layer of connective tissue. Nowhere could any invasive growth of the tumour cells into the capsule be seen.

The pathological-anatomical diagnosis was adrenal cortical adenoma. The structures of the tumour with its rather highly differentiated patterns and the absence of any obvious polymorphism and mitoses as well as invasive growth into the tumour capsule are arguments against it being malignant.

The testicles showed a normal number of tubules. They were lined by normal Sertoli cells, in some parts by a widened hyaline band. The tubules did not contain any spermatozoa, spermiogenesis was absent and spermatogenesis incomplete. The Leydig cells were morphologically normal and not increased in number.

The breast tissue showed hyperplasia of the periductal stroma and duct epithelium with proliferations into the widened ducts and acini. Some secretion in the ducts. No signs of malignancy.

The liver showed a normal distribution of cells and fat. Signs

of stasis and hemosiderosis but no fibrosis or cellular damage. The kidneys showed only slight changes of the smaller arteries as in arteriosclerosis but were otherwise normal. The pancreas was normal and had the usual number of normal islets.

*Survey of findings.* A 59 years old male showed bilateral gynecomastia, hypertension, decreased dextrose tolerance and increased tolerance to insulin. He excreted between 250 and 500 I. U. of oestrogenic hormone, less than 20 M. U. of gonadotrophic hormone, and 6.0 mg. of 17-ketosteroids per day. The liver function tests were normal. The pathological-anatomical findings were: a marked bilateral hyperplasia of the adrenal cortex, a very large cortical adenoma, pituitary changes resembling those seen in virilism, only minor changes in the liver, and inhibition of spermiogenesis in the testicles.

## DISCUSSION

There was certainly some relation between the gynecomastia in this case and the marked adrenal cortical hyperplasia and very large adrenal cortical adenoma. It is known that increased secretion of oestrogenic hormone may occur in cases with an adrenal cortical tumour (*Simpson & Joll, 1938, Roholm & Teilum, 1942*). Growth of the breasts and atrophy of the testicles are known effects of oestrogens in the male. As has already been mentioned, gynecomastia has previously been described in adrenal cortical cancer and adenoma. No relation between adrenal cortical hyperplasia and gynecomastia has so far definitely been proved, even though such a relation has been theoretically considered. (*Broster & Vines, 1933, Glass & Bergman, 1938*).

The simultaneous occurrence of an adrenal cortical adenoma and bilateral cortical hyperplasia in this case is noteworthy.

According to the literature it is usual to find the contralateral adrenal gland atrophic with an adrenal cortical tumour (*Kenyon, 1947*). Bilateral adrenal cortical hyperplasia suggests a central origin, i. e. an increased stimulation from the anterior lobe of the pituitary gland (see *Mellgren, 1945*).

Cytological changes occurred in the anterior lobe of the pituitary gland of the present case. These were of the same type as those previously described by *Mellgren* (1942, 1945) in virilism: i. e. hypertrophic amphophilic cells with foamy cytoplasm and ill defined cell margins. However, it is so far not known whether the occurrence of these cell types throws any light on the functional condition of the hypophysis.

Besides gynecomastia the patient also showed hypertension. The hypertension might in this case, of course, be a separate phenomenon, but it is interesting to note that hypertension has been described in cases of adrenal cortical hyperplasia or adenoma (for literature, see *Bergstrand*, 1947), and also that hypertension has been produced by the administration of adrenal cortical hormone, chiefly desoxycorticosterone acetate (for literature, see *Luft & Sjögren*, 1949).

The patient also showed a decreased dextrose tolerance and a relative insulin resistance. According to the modern conception of the significance of the adrenal cortex in carbohydrate metabolism, an increase of the »sugar-hormone« of the cortex helps to bring about a decrease of the dextrose tolerance and an increased tolerance to insulin (*Soskin & Levine*, 1946, *Ingle*, 1949).

Thus, there occurred in the present case of gynecomastia, hypertension and a disturbed carbohydrate metabolism, all symptoms that might be connected with an adrenal cortical hyperfunction. A change of the secondary sex characters in connection with hypertension, and a decreased dextrose tolerance is also found in Cushing's syndrome, where adrenal cortical hyperplasia is usually found (see *Luft*, 1944). The symptoms in Cushing's syndrome are mainly related to a disturbed interrelation between the anterior lobe of the pituitary gland and the adrenal cortex. Gynecomastia, however, has not been described in Cushing's syndrome. In the present case the clinical picture was completely different from that found in Cushing's syndrome. *Leth Pedersen* described in 1948 a case with virilism, hypertension and a decreased dextrose tolerance in a woman with adrenal cortical adenomas and a highly in-

creased excretion of 17-ketosteroids; i.e. a case in many respects similar to ours, but showing virilism instead of gynecomastia, and an increased excretion of 17-ketosteroids instead of oestrogens.

### SUMMARY

A case is reported (male, 59 years old) with gynecomastia, hypertension, decreased dextrose tolerance and a relative insulin resistance. The patho-anatomical findings consisted of considerable bilateral adrenal cortical hyperplasia, a very large adrenal cortical adenoma, and pituitary changes similar to those found in virilism.

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## SEXUAL INFANTILISM WITH RUDIMENTARY OVARIES

BY

KURT KAIJSER

The purpose of this paper is to present in detail the clinical findings in, and treatment of, a number of cases of sexual infantilism with rudimentary ovaries.

Such cases have often been previously described under the diagnosis of hypophyseal infantilism, without an adequate investigation of the pathology of the disease. In 1942 *Albright et al.* and *Varney et al.* showed that different forms of sexual infantilism could be differentiated by hormonal titration of the patient's urine.

### PREVIOUS CASES

The syndrome appears to have been described by seven authors as an occasional finding at operation, namely *Kermanner* (1912), *Ranson* (1913), *Sellheim* (1924), *Baer* (1927), *Kuliga* (1930), *Meyer* (1931), and *Goldwasser* (1933).

Thirteen cases have been found at post mortem examination and have been described by *Olivet* (1923), *Schultze* (1923), *Randerath* (1925), *Schürmann* (1927), *Herxheimer* (1929), *Rössle & Wallart* (1930), *Priesel* (1931), *Pela* (1935), *Pich* (1937—38), *Tronci* (1938), and *Sharpey-Shafer* (1941).

The following authors have given detailed clinical reports

of seventeen cases without, however, giving any information as to the condition of the ovaries: *Funke* (1902), *Nielsen* (1934), *Hess Thaysen* (1934), *Turner* (1938), *Höjer* (1945), *Rossi* (1945), *Hamne* (1948), and *Kaijser* (1948).

Since 1942 some sixty cases with a complete clinical examination and hormonal titration, together with a biopsy of the ovaries in a few cases, have been described by *Varney et al.* (1942), *Albright et al.* (1942), *Schneider & McCullagh* (1943), *Shereshevski* (1944), *Wilkins & Fleischmann* (1944), *del Castillo et al.* (1947), *Lisser et al.* (1947), *Greenblatt & Nieburgs* (1948), and *Goldman et al.* (1949).

It would thus appear that altogether over a 100 cases have been reported.

### SYMPTOMATOLOGY

The symptomatology which most of the cases described show is as follows:

1. Bodily under-development.
2. Primary amenorrhea.
3. Absence of breast development. Infantile external genitalia and hypoplastic uterus.
4. Small rudimentary ovaries, often in the form of a small, thin, hard, fibrous whitish string at the site of ovaries.
5. Absence or scanty growth of pubic and axillary hair. Often a scanty growth of hair first appears after the age of 15 years or so.
6. A marked increase of gonadotrophic hormone in the urine.
7. A marked decrease of oestrogenic hormone in the urine.
8. A somewhat diminished or normal amount of 17-ketosteroids in the urine.
9. A somewhat delayed or normal skeletal development as well as a manifest osteoporosis in the skeleton.
10. Moderate increase in blood pressure.
11. Presence of one or more congenital anomalies such as short neck, webbing of the neck, cubitus valgus, heart disease (e. g. coarctation of the aorta).

## MATERIAL

*Lisser* and his co-workers (1947), who have described 25 of their own cases, are of the opinion that this syndrome is probably not so rare as was previously believed. Support for this opinion may possibly be found in the fact that in the town of Umeå (16000 inhabitants) and its immediate surroundings in the northern part of Sweden, I have been able to collect as many as six such cases.

*Case No. 1.* L.L., 43 years of age. Sister of Case No. 2 and aunt of Case No. 3. Height 132 cm. Primary amenorrhea. Insignificant breast development after the age of 30. Somewhat earlier a scanty growth of hair appeared on the pubis and axillae. Virgin. External and internal genitalia hypoplastic. Ovaries not palpable. Intelligence test (Wählen's method): = 8—9 years. Roentgenogram of the heart and the chest: normal. Blood pressure 200/100 mm. of mercury. Insulin tolerance test (0.1 I. U./kg. body weight) normal. Hormone titration in urine: 17-ketosteroids = 21 mg. in 24 hours. Gonadotrophin: more than 80, less than 165 M. U. per litre. Oestrogen: less than 25 M. U. in 24 hours.\*) No biopsy carried out. The diagnosis can hardly be open to doubt in view of the typical appearance, her relationship to cases 2 and 3 and the high gonadotrophin content of the urine. The patient refused any form of treatment.

*Case No. 2.* A.L., 28 years of age. Sister of Case No. 1 and aunt of Case No. 3. Height 136.5 cm. Primary amenorrhea. Scanty growth of pubic hair from the age of 18. No growth of axillary hair. External and internal genitalia markedly hypoplastic. Ovaries not palpable. Virgin. Roentgenogram of the heart and the chest: normal. Blood pressure 150/100 mm. of mercury. Slight osteoporosis in spinal column and in the bones of the hands and feet. Insulin tolerance test: normal. Hormone titration in urine: 17-ketosteroids: 25 mg. in 24 hours. Gonadotrophin: 40 M.U. per litre. Oestrogen: less than 25 M. U. in 24 hours. No biopsy carried out. The diagnosis

\*) The author's normal values for female subjects are the following:

Age	17-ks/24 hrs.	Gon./l.	Oestrogens/24 hrs.
4—10	0—8 mg.	< 40 M. U.	< 25 M. U.
10—15	8—16 „	< 40 „	< 25 „
> 15	ca. 16 „	< 40 „	> 25 < 125 „

can be regarded as definite in view of the typical appearance, her relationship to Cases 1 and 3 and because of the low oestrogen content. The patient refused any form of treatment.

*Case No. 3.* S.D. Aged 15 years. First cousin of Cases 1 and 2. Has never menstruated.

*Physical Examination:* Height: 126 cm. (—34.2 cm.). Weight: 30.2 kg., (+ 5.8 kg.). Definite cubitus valgus. Short neck with slight webbing.

No mammary development. Nipples very small and partly sunken. External genitalia small and signs of puberty absent. A small, atrophic uterus can be palpated per rectum. No palpable resistance at the sides. Virgin. No pubic or axillary hair. Electrocardiogram normal. Roentgenography revealed an isolated dextrocardia. Blood pressure 150/115 mm. of mercury. Skeletal age normal. Slight osteoporosis. Blood calcium 9.3 mg. per cent. Blood phosphorus 2.1 mg. per cent.

Intelligence quotient 62 (Binet-Simon method).

*Exploratory laparotomy:* The uterus only as large as the tip of the little finger. Both tubes somewhat smaller than the normal size for her age. At the site of each ovary there is a white cord about two cm. long and a few mm. in width. The cord is smallest at the uterine end which forms a drawn out point. (A small part of the cord was excised for microscopic examination).

*Histological examination:* (C. W. Lundquist). The tendon-like cord consists of fibromatous connective tissue containing numerous large thin-walled, wide blood-filled vessels. The epithelial covering is a single layer of low cubical epithelium. There are no signs of primary follicles in the cortex of the rudimentary ovary (see Fig. 1 below).

*Diagnosis:* Made by biopsy of the ovaries. In this case attention is drawn to the patient's typical appearance, her relationship to cases 1 and 2 and the high gonadotrophin content found on one occasion (see below).

*Treatment:* For fourteen days after the operation the patient received altogether 27 mg. of oestradiol monobenzoate intramuscularly and 9 mg. of oestrone in tablet form orally. At the same time she was given an injection in the gluteal muscle of a suspension of crushed, fresh ovaries taken from a sexually mature calf.

Following this massive initial hormonal treatment, the patient was told to take oestradiol tablets by mouth (Follicyclin Ciba) in doses varying from 2 to 4 mg. a day. In addition she was given, an intramuscular injection of 10 mg. oestradiol monobenzoate (Follicyclin-B-Crystal ampoules), at intervals of 4 weeks, the intention being to form a depot of the preparation. This type of treatment was continued during the following year.

*Laboratory data:* (Unfortunately hormone titration of the urine was carried out for the first time after the above mentioned treatment had been started).



*Fig. 1.*

Microphotograph of ovary.  $\times 120$ .

Upper picture: Case 6 at age of 12. Diagnosis: rudimentary ovaries.

Middle picture: Girl aged  $5\frac{1}{2}$  years. Diagnosis: normal ovaries.

Lower picture: Case 3 at age of 15. Diagnosis: rudimentary ovaries.

One month after the operation: 17-ketosteroids: 17 mg. in the urine in 24 hours. Gonadotrophin: more than 80, less than 165 M. U. per litre. Oestrogen: more than 25, less than 125 M. U. in 24 hours.

*Result of continued treatment:* Four months after treatment was started, the girl had grown 2 cm. (height 128 cm.). Her nipples had become larger and were more pigmented. There was a slight growth of pubic hair.

After 7 months her height had increased 5 cm. and was 131 cm. She had now a normal mammary gland and almost normal distribution of pubic hair.

After 9 months she had a slight menstrual flow lasting for 15 days.

During the 11th and 12th month following the treatment this bleeding was fairly regular and lasted for 7 days.

The combined treatment consisting of one single intramuscular injection of crushed calf-ovaries and continued oral and parenteral administration of oestrogen thus brought about such a degree of maturity in the genital organs of this 15 year-old girl that a moderate development of her breasts and genital hair growth occurred and a scanty but regular menstrual flow appeared.

The short period of observation lasting for one year does not allow of any definite opinion regarding the prognosis.

*Case No. 4.* M. S. 38 years of age. Height 134.5 cm. Short neck. Definite cubitus valgus. Primary amenorrhea. No mammary development. Very small nipples. Very scanty growth of pubic and axillary hair. External and internal genitalia hypoplastic. Ovaries not palpable. Virgin. Blood pressure 135/85 mm. of mercury. Mental development normal. Insulin tolerance test: normal.

Hormone titration in the urine: 17-ketosteroids: 21 mg. in 24 hours. Gonadotrophin: more than 80 M. U. per litre. Oestrogen: less than 25 M. U. in 24 hours. No biopsy. The diagnosis can be considered as beyond doubt in view of the typical appearance, the high gonadotrophin and low oestrogen content in the urine. The patient refused all forms of treatment.

*Case No. 5.* K. L. 14 years old. Primary amenorrhea. *Physical examination:* Height 133 cm. (—26 cm.). Weight 33.5 kg. (+4.8 kg.). Definite cubitus valgus. Fairly short neck but no webbing. No mammary development. Nipples small but not sunken. External genitalia small. Virgin. A small narrow uterus 3—4 cm. long, can be felt per rectum. No ovaries could be palpated with any degree of certainty. No pubic or axillary hair. Physical examination of the circulatory system reveals normal findings. Slight right ventricular preponderance in electrocardiogram. Skeletal age normal. Slight osteoporosis in bones of hands and feet. Mental development normal.

*Laboratory data:* Blood calcium 10.2 mg. per cent. Blood phosphorus 6.1 mg. per cent. Hormone titration in urine (on two occasions at an interval of ten months): 17-ketosteroids: 30 mg. in 24 hours.

Gonadotrophin: less than 40 M. U. per litre.

Oestrogen: less than 25 M. U. in 24 hours.

*Diagnosis:* The typical appearance together with the high gonadotrophin content (see the value below) seem to make the diagnosis definite.

*Treatment.* During a period of about one month the girl received a series of injections of chorionic gonadotrophin (Physex, Leo), altogether 22620 I. U.

There was no development of the mammae or growth of pubic hair following this treatment. Laboratory data ten months after the beginning of the treatment: Gonadotrophin: more than 80 M. U. per litre. Oestrogen: less than 25 M. U. in 24 hours.

For at least a year after this the treatment was suspended. It was started again when the patient was about 15½ years old and when she still had a height of 136 cm. and no signs of puberty.

The new treatment was planned so that the patient, for the first three months, was given one intramuscular injection per week of 1 mg. of oestradiol monobenzoate (Dimenformon, Pharmacia). The tablet dose was raised to 0.6 mg. a day during the second month and to 0.9 mg. during the third month. From the beginning of the fourth month of treatment, the dose was still further increased to 1 mg. of oestrone daily (Menformon) by month.

As a result of the four months' treatment described above, her height has increased from 136 cm. to 140 cm. her breasts have shown a moderate development (without any simultaneous growth of pubic hair) and for two days every month she has had a very slight menstrual flow. The patient has now been treated in this way for over ten months.

*Case No. 6.* G-M. E. 10 years of age. Primary amenorrhea.

*Physical examination:* Height 111 cm. (— 32 cm.). Weight 18.7 kg. ( $\pm$  0 kg.). Definite cubitus valgus. Very short neck with obvious webbing. No mammary development. Nipples very small and sunken. External genitalia hypoplastic (for internal genitalia see below). Absence of hair on pubis and axillae. Roentgenogram of heart normal. Electrocardiogram normal. Blood pressure 135/85 mm. of mercury. Normal skeletal age and moderate osteoporosis.

Intelligence quotient 90 (Terman and Merrill's method).

*Laboratory data:* Blood calcium 10 mg. per cent. Blood phosphorus 2.9 mg. per cent. Insulin tolerance test: normal.

*Hormone titration in urine:* 17-ketosteroids: 15 mg. in 24 hours. Gonadotrophin: less than 40 M. U. per litre. Oestrogen: less than 25 M. U. in 24 hours.

*Exploratory laparotomy:* There is only slight development of the



uterus which has the thickness of a lead pencil. The tubes appear to be underdeveloped for the age. No ovaries can be observed; at the site of these there is only a cord a few cm. long and a few mm. broad which is composed of a white, shining tissue. (A small piece of this was excised for further investigation).

*Histological examination:* (C. W. Lundquist): The piece of rudimentary ovary which was examined showed ovarian tissue in which it was possible to demarcate a cortical zone without any primary follicles, and a medullary zone with numerous wide, fairly thin-walled blood vessels. On the surface one sees an ovarian epithelium of usual appearance. (see Fig. 1, top picture).

*Treatment:* Treatment was commenced when the girl was 12 years old and had a height of 114 cm. A suspension from both ovaries of a 7 months old calf was prepared and injected deep intramuscularly. Subsequently she received an intramuscular injection of 10 mg. oestradiol monobenzoate (Follicyclin B-crystal ampoules) about once a month and oestradiol tablets (Follicyclin-Ciba) by mouth at the rate of 1 mg. a day during the first 2 months, 2 mg. a day during the next two months and thereafter 4 mg. a day.

*Result of treatment:* When the patient was examined 3 months after the beginning of the treatment, she had grown 3 cm. in height which was exactly equivalent to the amount she had grown during the two previous years. Her mamillae had developed and one could feel under them a definite mammary glandular tissue as large as a hazel nut. There was a growth of fine hair on the pubis. She had a scanty menstrual flow lasting for two days.

When she was examined 6 months after the beginning of treatment her height had further increased by almost 3 cm. There was no further development of the breasts or growth of pubic hair.

## DISCUSSION

The syndrome of rudimentary ovaries and infantilism offers quite a number of interesting points for discussion.

The question of infantilism has been particularly stressed. It is doubtful whether one can explain infantilism as being merely the result of a failure of hormonal activity due to ovarian aplasia.

The presence of other degenerative changes such as congenital heart disease, webbed neck, etc., would suggest that the whole syndrome points to a primary degenerative injury taking place simultaneously in different organic systems. On

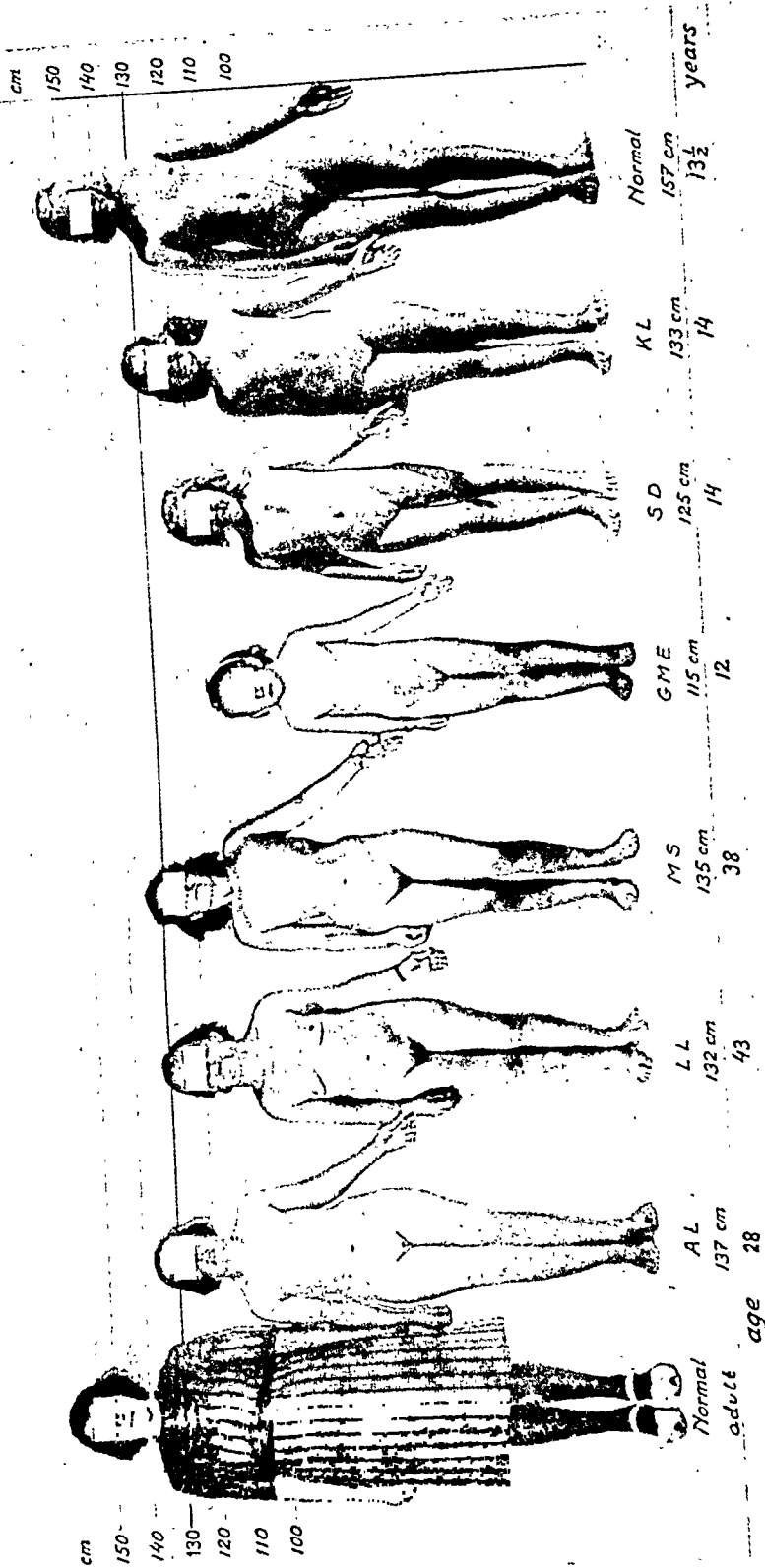


Fig. 2.

The 6 cases of rudimentary ovaries as compared with an adult, normal woman (at the extreme left) and with a girl of 13½ years (at the extreme right) of normal height, weight and development in keeping with her age.

Table 1.

Summary of some of the essential findings in the 6 cases described.

Date	Case Name	Age Years	Heightage Years	Height cm.	Weight kg.	Mammary development	Genital development	Hair P. A.	FSH M. U./l.	Oestrogens M. U. per 24 hours	17-ks. mg. per 24 hours	Biopsy
1948 3./11.	1 L. L.	43	9	132	39.6	(+)	—	+ —	>80 <165	<25	21	—
1948 8./11.	2 A. L.	28	10	137	43.5	—	—	(+) —	<40	<25	25	—
1948 25./6.	3 S. D.	15	9	126	30.2	—	—	— —				+
1948 3./8.	S. D. 1)								>80 <165	250	17	
1948 27./10.	4 M. S.	38	10	135	37.7	—	—	(+)(+)	>80	<25	21	—
1947 18./8.	5 K. L.	14	9	133	33.5	—	—	— —	<40	<25	30	—
1948 23./10.	K. L.	15	10	135	36.3	—	—	— —	>80	<25		—
1948 3./2.	6 GM.E.	11	5	111	18.7	—	—	— —				—
1948 14./9.	GM.E.	12	6	115	20.4	—	—	— —	<40	<25	15	+

1) After oestrone treatment.

P. = pubis. A. = axilla. MU. = mouse units. FSH. = follicle-stimulating hormone. 17-ks. = 17-ketosteroids.

the other hand, in those cases in which such changes cannot be demonstrated in other organs, the genesis of the disease might perhaps be dependent on premature degeneration of the ovaries alone, followed by sexual underdevelopment. It is note-

Table 2.

Summary of some of the essential findings in the 6 cases described.

Case Name	Age Years	Skeletal development	Osteoporosis	Hypertension	Insultest	Electro-cardiogram	Intelligens-quotient	Remarks
1 L. L.	43	N	+	+	N	N	60	Slight webbing of the neck
2 A. L.	28	N <sup>1)</sup>	+	+	N	(N) <sup>2)</sup>		Slight webbing of the neck
3 S. D.	15	N	+	+	(N) <sup>3)</sup>	N	62	Evident webbing of the neck
4 M. S.	38	N	—		N	N	N	
5 K. L.	14	N	+			N	N	
6 G.M.E.	10	N	+	+	(N) <sup>3)</sup>	N	90	Evident webbing of the neck

1) Remaining epiphyseal lines.

2) Slight myocardial changes.

3) Insignificant decrease in blood sugar.

N. = normal conditions.

worthy that a moderate degree of bodily underdevelopment is a characteristic feature in many cases.

Finally I would point out that the presence of cubitus valgus, which many authors have emphasized as being especially typical of this syndrome, is probably associated with the general bodily underdevelopment rather than with the infantilism per se.

One detail of interest is the condition of the thymus in these naturally castrated girls. One might almost expect to find the thymus hyperplastic. Nothing of the kind, however, could be demonstrated.

### *Treatment.*

As it would appear that the essential feature of the disease is an absence of ovarian hormone, it seems reasonable to employ substitution therapy in the form of oestrone and progesterone.

The ideal treatment would be to transplant an ovary from a normal woman into the patient at the site of the hypoplastic ovarian cords. However, it has not been demonstrated with any degree of certainty that such an ovary survives after such a transplantation. It is more probable that it undergoes resorption after a short period, thus producing a massive hormonal stimulus.

A powerful hormonal action such as this, should also be obtained by injections of crushed ovaries obtained from some animal such as a calf. An injection of this nature was accordingly given to cases 3 and 6.

There are numerous excellent, synthetic oestrogen preparations obtainable. The primary aim of the treatment is to produce maturity of the patient's genitalia. By means of a continued administration of an oestrogenic preparation by injection or by mouth, one can achieve a certain degree of maturity. The cases treated are so few in number and of such varying ages that one has no definite knowledge as to the amount of the different preparations required. The treatment of the patients is given in more detail in the case histories described above.

In the second place it is desirable, by means of treatment, to retain the maturity of the genitalia and in addition to initiate and maintain a normal menstrual cycle. A result such as this ought to assist in promoting not only the physical but also the psychological well-being of the patient.

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Oestrone and progesterone preparations have been kindly placed at our disposal by Messrs. Ciba Ltd. (Follicyclin, Lutocyclin) and Messrs. Pharmacia Ltd. (Di-menformon, Menformon, Progestin).

## SUMMARY

Six cases of sexual infantilism with rudimentary ovaries, of ages varying from 10 to 40 years, have been observed within a fairly limited region in Northern Sweden; three of them were closely related. In all six cases hormone titration of the urine was carried out and showed, in most cases, a high value for gonadotrophin and, at the same time, a low oestrogen content.

A clinical report, emphasizing the most prominent symptoms, is given in all the cases. Biopsy by means of laparotomy together with a histological examination of a piece of the rudimentary ovaries was carried out in two cases.

Three cases refused treatment on account of their advanced age.

Two cases were given intramuscular injections of the crushed ovaries of calves. In addition, these two patients and one other were given repeated doses of oestrogen. All three showed an increase in bodily height, a certain degree of maturity of their genitalia and a scanty menstrual flow.

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## GIANT GROWTH OF RAT FETUSES PRODUCED EXPERIMENTALLY BY MEANS OF ADMINISTRATION OF HORMONES TO THE MOTHER DURING PREGNANCY\*)

BY

GÖSTA T. HULTQUIST and BENGT ENGFELDT

In an investigation on the effect of diabetes on pregnancy and on the offspring in rats, *Hultquist* (1948) observed an increase in the weight of the offspring at birth in about 15 per cent of the litters. If diabetes was produced by subtotal excision of the pancreas in the middle of pregnancy, an increase in the birth weight of the offspring was obtained in about 40 per cent of the litters. The increased birth weight was due to genuine giant growth with increased length and enlargement of the organs, rather than to increased accumulation of fat. Moreover, many of the giant offspring had dropsy. The mortality rate was high, 82 per cent, and many of the giant fetuses were born dead and macerated.

The endocrine organs of the giant offspring showed a characteristic picture. Adrenals, testes, parathyroids, thymus, epiphysis and the islets of Langerhans showed hyperplasia, which was most marked in the testes and adrenals and least marked and not quite significant in the thymus and islets of Langerhans. The thyroid gland and the ovaries, however, were

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\*) From a paper read on the Staff Meeting of the Caroline Hospital, April 2nd 1949.



not enlarged. Apart from enlargement of the posterior lobe, the hypophysis showed no definite quantitative changes; the anterior lobe, however, showed a more differentiated cell picture with an increased number of chromophilic cells, particularly of basophiles, as compared with the control animals.

The marked correlation between giant growth and the changes in the endocrine organs described above suggests that they are related. The facts indicate that they are due to an effect transmitted from the mother to the fetus during pregnancy. This effect could be brought about in one of two ways. There may be a direct effect which causes both the giant growth and the changes in the endocrine organs, as parallel phenomena. Alternately, the changes in the endocrine organs may be the direct result of the effect exerted by the mother, and the giant growth might be secondary to the endocrine changes. *Hultquist's* results failed to show which is the true explanation. Judging from the nature, and in certain cases also the incidence and degree of the changes in the endocrine organs, the assumption that the giant growth and the endocrine changes are parallel phenomena appears to be the more probable.

It is a reasonable assumption that the influence of the mother on the fetus is of a hormonal nature. On the basis of this assumption we have attempted to answer the following questions in the present experiment:

1. *Can giant growth be produced in fetuses by the administration of hormones to the mother during pregnancy?*
2. *Can changes in the endocrine organs, similar to those in the giant offspring of diabetic rats, be produced in fetuses by the administration of hormones to the mother during pregnancy?*

## MATERIAL AND TECHNIQUE

The experiments were performed on rats. In the first place anterior pituitary hormones were used, viz. growth hormone, corticotrophic and thyrotrophic hormones, then various go-

nadotrophic hormones were tried. The hormones were as a rule given in increasing doses during the whole of pregnancy, which lasts for about 22 days in rats. Experiments have also been carried out with a number of other hormones. These latter experiments, however, will not be reported here. Table 1 shows the preparations used and the dosage given to each animal during pregnancy.

Table 1.

Growth hormone, Phyol (Alfred Benzon, Copenhagen)	80—120 U.
Antuitrin G (Parke, Davis)	120—180 U.
Pregnant mare serum gonadotrophin, Antex (Leo)	80—110 I. U.
Chorionic gonadotrophin, Physex (Leo), Gonadex (Leo), Pregnyl (Pharmacia)	80—150 I. U.
Corticotrophic hormone (Nordiska Organon)*)	800—7000 U.
Thyrotrophic hormone (     "     "     )*)	100—200 U.**)

## RESULTS

As seen from Table 2, statistically significant giant growth occurs in the offspring, when growth hormones (Phyol and Antuitrin G), chorionic gonadotrophin and thyrotrophic hormones are administered to the mothers during pregnancy. In all these cases it was observed that the period of pregnancy was prolonged by 1—4 days. The experiments with chorionic gonadotrophin were only successful if the hormone was administered in a concentrated form during the last 3—5 days of pregnancy. When the same dosage was distributed over the entire period of pregnancy parturition took place at the normal time. Prolongation of the period of pregnancy may, *a priori*, be assumed to result in enlarged offspring. In some of the experiments, therefore, the mother was killed at full term, 22 days after the appearance of the vaginal plug, and

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\*) For the preparations received we are indebted to Nordiska Organon, Stockholm.

\*\*) Junkmann-Schoeller and Heyl-Laqueur respectively.

Table 2.  
Birth weight of rat offspring, whose mothers had received hormones during pregnancy.

Mothers weight		150—195					200—245					250—295					
Birth weight of rat offspring (gm.)																	
	Number (n)	Min.	Max.	Average (m)	$\sigma$	$\epsilon_m = \frac{s}{\sigma}$	Number (n)	Min.	Max.	Average (m)	$\sigma$	$\epsilon_m = \frac{s}{\sigma}$	Number (n)	Min.	Max.	Average (m)	$\epsilon_m = \frac{s}{\sigma}$
Controls	327	3,6	6,0	5,1	$\pm 0,43$	$\pm 0,024$	262	3,8	6,2	5,2	$\pm 0,48$	$\pm 0,030$	35	4,4	6,7	5,3	$\pm 0,49$
Growth hormone (Phylol) prolonged pregnancy	65	5,0	7,6	6,0	$\pm 0,58$	$\pm 0,072$	6	5,7	6,4	6,1	$\pm 0,25$	$\pm 0,102$					
					$\pm \text{diff} = 12 \times \epsilon \text{ diff}$						$\pm \text{diff} = 8 \times \epsilon \text{ diff}$						
Phylol full term	51	3,6	8,1	5,6	$\pm 1,00$	$\pm 0,140$	27	4,5	6,1	5,4	$\pm 0,45$	$\pm 0,087$					
					$\pm \text{diff} = 3,5 \times \epsilon \text{ diff}$						$\pm \text{diff} = 2,1 \times \epsilon \text{ diff}$						
Growth hormone (Antuitrin G) prolonged pregnancy							15	5,2	7,0	5,7	$\pm 0,49$	$\pm 0,127$					
											$\pm \text{diff} = 3,8 \times \epsilon \text{ diff}$						
					</												



the offspring was weighed. In these cases an increase in the birth weight was observed in the offspring of animals treated with growth hormone but not in those treated with chorionic gonadotrophin.

The results indicate that prolongation of pregnancy tends to increase the birth weight. Enlarged offspring at full term, however, may also be produced after treatment of the mother with growth hormone. Thus, prolongation of pregnancy is evidently not the sole cause of the giant growth, but is apparently a contributory factor.

The mortality rate in the litters of giant offspring was high and in many cases the fetuses showed maceration and dropsy, similar to the giant offspring of diabetic mothers. After administration of Phylol producing prolonged pregnancy, the mortality rate among the offspring was nearly 50 per cent and after administration of Antuitrin G, 40 per cent.

Thus, the question as to whether it is possible to produce giant growth in rat fetuses by means of the administration of hormones to the mother during pregnancy may be answered in the affirmative. In this respect the most marked effect seems to be produced by growth hormone.

An increase in the birth weight of young rats, produced by the administration to the mother of anterior pituitary lobe preparations containing growth hormone as the main factor, has been observed previously by *Teel* (1926) and *Hain* (1932). Experiments with Antuitrin G, *Sontag & Munson* 1934, and with Phyone,\*) *Watts* (1935), have also demonstrated an increase in the birth weight of young rats. *Snyder* (1934) and *Hoopes* (1934) have obtained similar results in experiments with chorionic gonadotrophin. In these experiments prolongation of the period of pregnancy was observed in many cases. Apparently, the endocrine organs are not studied in any of the above mentioned experiments.

The endocrine organs of the rat offspring were examined

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\*) Phyone is a relatively pure growth-hormone preparation of American origin.

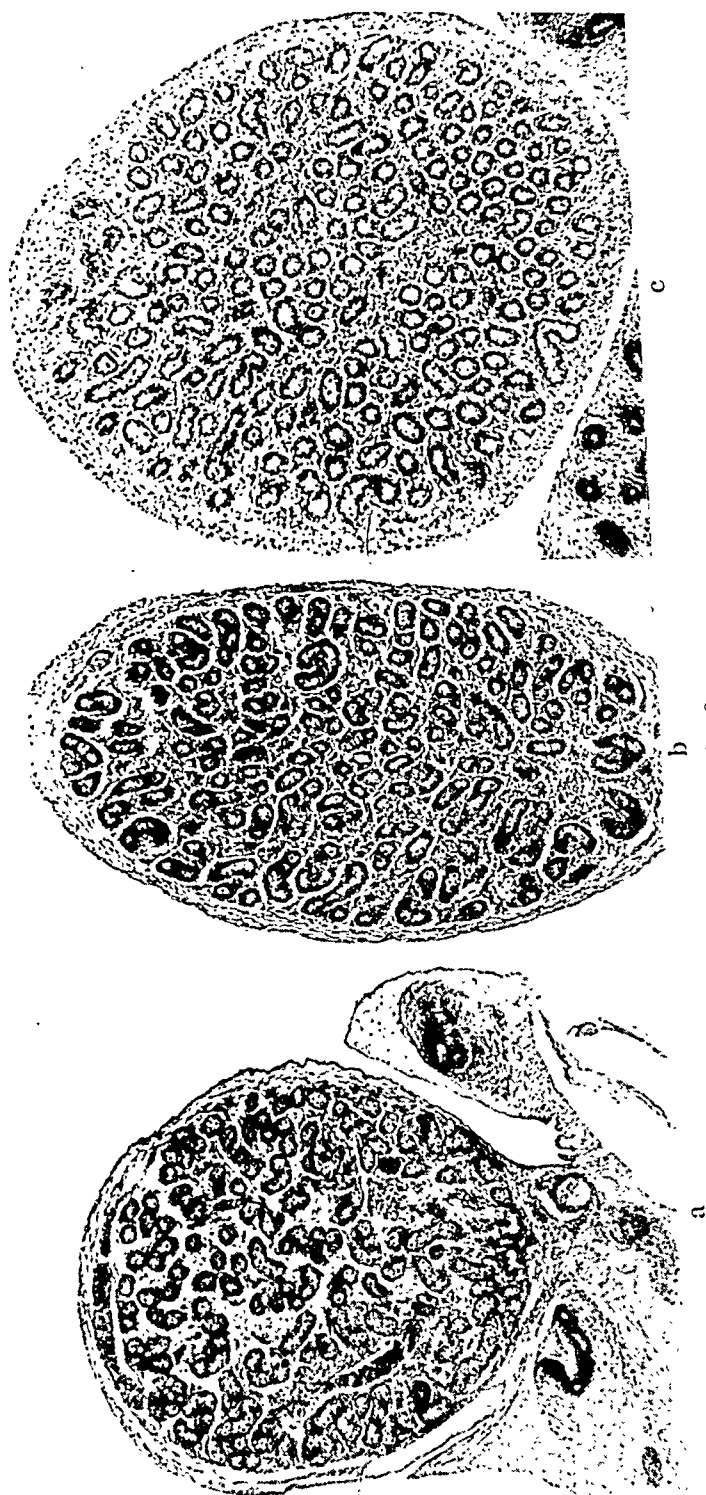
by serial section, and their volume was determined planimetrically after outlining in a projection apparatus. In the case of the giant offspring produced by the administration of growth hormone, the histological changes in the endocrine organs were similar to those characteristically found in the



*Fig. 1.*

Giant offspring (8.1 gm.) after administration of growth hormone to the mother during pregnancy, in comparison with normal offspring (5.1 gm.).

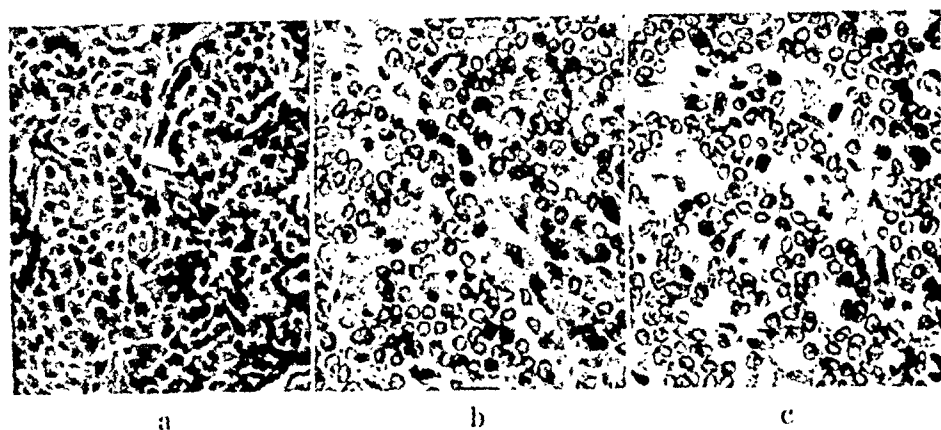
endocrine organs of giant offspring of diabetic rats. The adrenal cortex showed hyperemia and columnar formations of the cells in the zona fasciculata. The testes showed an increase in the size of the tubules, in many cases with formation of lumen in the centre and an increase in the number of interstitial cells (Fig. 2). In the parathyroids there was a marked increase in the size of the cell nucleus and the cytoplasm, and a cellular structure similar to that found in growing cells was observed. The parathyroids also showed greater vascularisation and hyperemia (Fig. 3). The anterior pituitary lobe presented a more differentiated cell picture than normal



*Fig. 2.*

- a. Testis of normal offspring. ( $\times 65$ ).
- b. Testis of giant offspring, whose mother had received growth hormone (Phyol) during pregnancy ( $\times 65$ ).
- c. Testis of giant offspring of diabetic mother. ( $\times 65$ ).

with an increased number of chromophilic elements, particularly basophiles. The thyroid showed a more compact structure with less distinct formation of follicles than in the controls. In the experiments with the other hormones changes



*Fig. 3.*

- a. Parathyroid gland of normal offspring. ( $\times 500$ ).
- b. Parathyroid gland of giant offspring whose mother had received growth hormone (Phyol) during pregnancy. ( $\times 500$ ).
- c. Parathyroid gland of giant offspring of diabetic mother. ( $\times 500$ ).

of this type were demonstrated only in a few organs. After administration of chorionic gonadotrophin to the mother, the parathyroids of the offspring, for instance, showed the same type of changes as those observed after the administration of growth hormone.

The quantitative values for the endocrine organs of the offspring are recorded in Table 3. As seen from the table, the quantitative values for the giant offspring after administration of growth hormone are on the whole in agreement with the values for the giant offspring of diabetic mothers. Only the parathyroids and the epiphysis show no statistically significant quantitative changes (the difference being respectively 2 and 1.4 times the standard error of the difference).

The experiments with thyrotrophic hormone showed hyperplasia only of the adrenals and the islets of the pancreas, in



spite of the fact that the adrenals as well as the parathyroid and thyroid glands showed qualitatively some changes of the same type as those seen in the giant offspring of diabetic rats.

With regard to other hormones used, hyperplasia was observed in a few organs, but in no case were there the same general changes as those seen following administration of growth hormone.

Thus, the question as to whether changes in the endocrine organs, similar to those in diabetic offspring, may be produced in young rats by the administration of hormones to the mother, may also be answered in the affirmative as regards growth-hormone preparations.

The two different growth-hormone preparations used in the present experiments have shown very different effects. The changes are definite in the offspring of mothers that had received Phylol, but doubtful after the administration of Antuitrin G. The degree of purity of the hormone preparations is important, and according to the manufacturers, Phylol is very nearly as pure as the growth-hormone preparations produced by *Evans* and co-workers, while Antuitrin G contains small amounts of thyrotrophic and gonadotrophic hormones. The thyrotrophic hormone available to us is not pure and contains, according to information, supplied by Dr. F. Paulsen, Nordiska Organon, Stockholm, at least gonadotrophin.

In the experiments that we have carried out up to the present, we have found that giant growth and the changes typical of the giant diabetic offspring are both evident only after the administration to the mother of preparations whose main content is growth hormone. Thus, the experiments indicate that the hormonal factor which likely causes giant growth in offspring of diabetic rats and characteristic changes in the endocrine organs of the offspring, is present particularly in the growth hormone preparation Phylol. So far it has not been possible to decide whether the factor is completely identical with the growth hormone, as the preparation used is not absolutely pure.





All the litters produced by mothers receiving growth hormone do not show giant growth. The appearance of the changes in the endocrine organs is not quite constant in the giant offspring and in a few cases the changes are not clearly demonstrable. As mentioned briefly above, it is therefore likely that other factors may contribute or that individual variations in the experimental animals used may influence the development of the giant growth.

Some of the animals treated with growth hormone were subjected to glucose tolerance tests at different stages of pregnancy, but the results were essentially the same as in the control animals. An interesting fact is that in rats, according to *Bennett* (1948) growth hormone does not cause changes in the urinary glucose but causes retention of nitrogen in hypophysectomised diabetic rats.

### DISCUSSION

With regard to the mechanism of the production of giant growth we can at the present stage only make tentative suggestions. There seem to be several possible explanations. The supposed hormonal factor, for instance, may be produced in the hypophysis of the mother and be transferred to the fetus via the placenta, or the factor may be produced in the placenta itself. Another possible explanation is that the hormonal factor affects the metabolism of the mother in the first instance, e. g. the metabolism of the fetus with e. g. retention of nitrogen and synthesis and accumulation of protein. On the basis of the results obtained so far, however, it is not possible to form a definite opinion on this subject.

### SUMMARY

Various hormone preparations (gonadotrophins, growth hormone, thyrotrophin, corticotrophin) were administered to pregnant rats.

Giant litters were produced, mainly in experiments in

which growth hormone preparations had been administered. These giant animals showed changes in the endocrine organs similar to those seen in giant litters of diabetic mothers. Gigantism was not solely due to prolongation of pregnancy.

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## AUGMENTATION OF CHORIONIC GONADOTROPHIN BY POLYVINYLPYRROLIDONE

BY

K. PEDERSEN-BJERGAARD and M. TØNNESEN

It has been repeatedly demonstrated that there are several biological differences between the actions of mare serum gonadotrophin and human chorionic gonadotrophin. As shown by *Hamburger & Pedersen-Bjergaard* (1938), mare serum gonadotrophin produces a similar response whether the hormone is administered in five doses during forty-eight hours or given in a single dose by subcutaneous or intravenous injection. In the case of chorionic gonadotrophin, however, the number of injections and the manner in which they are given are of considerable importance; in this respect chorionic gonadotrophin behaves as most other hormone preparations in aqueous solution.

Several factors might account for the difference between these two gonadotrophins, e. g. differences in the rate of destruction and excretion.

The possibility that mare serum gonadotrophin is absorbed slowly is confirmed by the fact that substances which augment hypophyseal gonadotrophin extracts by means of delayed absorption, such as zinc sulphate and inert proteins, do not augment mare serum gonadotrophin (*Saunders & Cole*, 1936; *Lein*, 1937; *Deanesly*, 1939). The latter author found

that if the absorption of mare serum gonadotrophin was delayed by the addition of zinc sulphate, the ovarian response was correspondingly decreased, as the total dose was probably no longer completely utilized during the 5-days' rat test. The significance of slow absorption must, however, be of very minor importance, as an intravenous injection is just as effective as a subcutaneous one (*Hamburger & Pedersen-Bjergaard, 1938*).

*Catchpole et al.* (1935) found that mare serum gonadotrophin is only slowly destroyed within the animal body and that the hormonal concentration was reduced by approximately one-half every twenty-six hours in the rabbit and by one-half every six days in the gelding. As far as is known, the rate of destruction of the gonadotrophin in rats has not been investigated, but it is very likely that slow destruction may be a cause of the similarity between the response obtained from single and from divided doses.

Slow destruction can be of significance only if little or none of the hormone is excreted. Whilst chorionic gonadotrophin is excreted in the urine of pregnant women, as well as after injections into man and into rabbits (*Parkes & White, 1933*), mare serum gonadotrophin is not excreted through the kidneys in pregnant mares or after injections into monkeys and rats (*Evans et al., 1933*, and *Hamburger, 1938*).

It is therefore reasonable to assume that non-excretion as well as slow destruction of mare serum gonadotrophin is the cause for the independence of the action of this gonadotrophin from the method of administration.

Among the substances known to cause a delayed absorption of hormones is polyvinylpyrrolidone. This substance was used clinically by *Lederer* (1949) in the treatment of diabetes insipidus with an extract of the posterior lobe of the hypophysis. The remarkable effectiveness of this treatment led us to investigate the effect of polyvinylpyrrolidone in combination with gonadotrophic hormones. The present paper is a report of our experiment with immature female rats and rhesus monkeys treated in various ways with pregnant mare serum hor-

mone and chorionic gonadotrophin alone or in combination with polyvinylpyrrolidone.

## MATERIAL AND TECHNIQUE

### *Animal material.*

The *rats* used were taken from our own laboratory stock, a breed of albinos which have been in-bred for many years. The date of birth of all the animals is known, and only animals twenty-six days old are used. At this age the average weight is from 35—45 gm. and the average weight of one pair of ovaries is 10 mg.

Four female *Macacus rhesus* monkeys weighing 3.8 kg., 2.9 kg., 2.7 kg., and 2.7 kg., respectively were used. The animals were immature, had never menstruated but, considering their weight, were almost sexually mature, since *Zuckerman* (1930) has reported that the first menstruation in *Macacus rhesus* monkeys occurs when the animals weigh about 3.3 kg.

### *Substances.*

*Pregnant mare serum gonadotrophin* (PMS). A highly purified preparation, »Antex 46114«, in the form of a dried powder, containing about 99 per cent of lactose, easily soluble in water and standardized in international units.

*Chorionic gonadotrophin*. A highly purified preparation, »Physex 490317«, in the form of a dried powder, containing about 99 per cent lactose, easily soluble in water, standardized in international units and of a purity as stated in the paper by *Madsen et al.* (1949).

*Polyvinylpyrrolidone* (PVP). We have used the preparation »Periston« Bayer, concentrated by evaporation to a suitable strength.

### *Methods of administration.*

In the *rats* the preparations were administered as single subcutaneous injections, or the total dose was given as 5 equal doses in the course of 48 hours.



The *monkeys* received one subcutaneous injection of PMS and 5 days later one subcutaneous injection of chorionic gonadotrophin.

#### *Solutions.*

The preparation were dissolved in such volumes of saline or of PVP solution that the total dose was contained in 1 ml., with one exception (Table 3 B).

#### *Vaginal smears.*

Vaginal smears from the rats were examined on the third, fourth and fifth day, altogether 5 smears being taken.

#### *Autopsy and operation.*

The *rats* treated with PMS were autopsied 100, 150, and 200 hours after the injection, the ovaries being removed, dissected and weighed while fresh. The rats treated with chorionic gonadotrophin were not killed, the criterion of response being vaginal cornification.

The *monkeys* were laparotomized under ether anaesthesia on the 11th day after the first injection, the ovaries were removed, weighed and examined histologically.

## RESULTS

### *A. Infantile rats.*

*Expt. No. 1. Pregnant mare serum gonadotrophin.* Altogether 180 rats were used for this experiment. They were all given a single subcutaneous injection. Half the animals were injected with the hormone in saline solution, the other half with the hormone dissolved in a 25 per cent solution of PVP. Three dose levels were examined: 10, 20 and 40 I. U. respectively, and within each group autopsy was performed 100, 150 and 200 hours after the injection. As seen from Table 1, the addition of PVP had no effect on the ovarian weight obtained in any of the experimental groups.

*Expt. No. 2. Chorionic gonadotrophin, divided dosage.*

Table 1.

Ovarian weight of infantile female rats treated with a single subcutaneous injection of mare serum gonadotrophin in aqueous solution and in a 25 per cent solution of polyvinylpyrrolidone.

Preparation	Dose (in I. U.)	Number of rats in each group	Average ovar. weight (in mg.) after:		
			100 hours	150 hours	200 hours
PMS in saline	10	10	41	34	26
» » »	20	10	81	57	57
» » »	40	10	108	95	64
PMS in 25 per cent PVP	10	10	43	22	20
» » » » » »	20	10	80	74	40
» » » » » »	40	10	121	99	77

Groups of 10 rats were treated with 0.3 and 0.6 I. U. either alone or in combination with a 3 per cent PVP solution. The percentage of positive vaginal smears (Table 2 A) was not altered by the addition of PVP.

*Expt. No. 3. Chorionic gonadotrophin, single subcutaneous injection.* Forty rats were treated with 0.3, 0.6, 1.2 and 2.4 I. U. of chorionic gonadotrophin in saline and an equal number of rats received the same doses of the hormone dissolved in a 3 per cent PVP solution. As seen from Table 2 B, the addition of PVP considerably increased the percentage of cornified vaginal smears. From dose-response curves it was calculated that a *hundred per cent augmentation* had taken place. A comparison of Table 2 A and Table 2 B shows that the single subcutaneous injection was one quarter as active as repeated subcutaneous injections when given in aqueous solution, but one-half as active when given in 3 per cent PVP solution.

*Expt. No. 4. Chorionic gonadotrophin and various concentrations of polyvinylpyrrolidone solution.* Three groups of 10 rats all received the same dose of hormone (0.4 I. U. as a single subcutaneous injection) but the concentration of PVP was varied. It is seen (Table 3 A) that the augmentation increases with the concentration of PVP. With 25 per cent a *five*

Table 2.

Percentage vaginal cornification in infantile female rats treated with chorionic gonadotrophin in aqueous solution and in solutions of polyvinylpyrrolidone.

*A. Dose divided in 5 subcutaneous injections.*

Preparation	Dose (in I. U.)	Number of rats	Per cent positive vaginal smears
Chorionic gonadotrophin	0.3	10	40
in saline	0.6	10	80
Chorionic gonadotrophin	0.3	10	40
in 3 per cent PVP	0.6	10	80

*B. Dose in one single subcutaneous injection.*

Preparation	Dose (in I. U.)	Number of rats	Per cent positive vaginal smears
	0.3	10	0
Chorionic gonadotrophin	0.6	10	0
in saline	1.2	10	30
	2.4	10	100
	0.3	10	10
Chorionic gonadotrophin	0.6	10	50
in 3 per cent PVP	1.2	10	100
	2.4	10	100

*hundred per cent augmentation* was obtained, as calculated from the dose-response curves.

*Expt. No. 5. Chorionic gonadotrophin in different amounts of a 25 per cent polyvinylpyrrolidone solution.* Five groups of 10 rats each received a single subcutaneous injection of 0.3 I. U. of chorionic gonadotrophin dissolved in different volumes of a 25 per cent PVP solution (Table 3 B). The augmentation obtained was insignificant until the volume was raised to 1 ml.

*Expt. No. 6. Intravenous injection of polyvinylpyrrolidone and subcutaneous or intravenous injection of chorionic gonadotrophin.* Three groups of 10 rats were injected intravenously with 1.2, 2.4 and 4.8 I. U. of chorionic gonadotrophin respectively and an equal number of rats were given the same hormone treatment together with 1 ml. of a 25 per cent solution

*Table 3.*

Percentage vaginal cornification in infantile female rats treated with one single subcutaneous dose of chorionic gonadotrophin in different amounts of polyvinylpyrrolidone solutions of different concentrations.

*A. Different concentrations of polyvinylpyrrolidone solution, 4 ml.*

Number of rats in each group	International units	3 per cent	10 per cent	25 per cent
10	0.4	20	50	80

*B. Different amounts of a 25 per cent polyvinylpyrrolidone solution.*

Numb. of rats in each group	International units	0.1 ml.	0.2 ml.	0.4 ml.	0.5 ml.	1.0 ml.
10	0.3	20	20	20	30	60

of PVP. As calculated from the figures in Table 4 A, a 50 per cent augmentation occurred.

The effects of subcutaneous injection of 0.6, 1.2 and 2.4 I. U. of chorionic gonadotrophin respectively into three groups of 10 rats were compared with the effects in an equal number of rats given 0.3, 0.6 and 1.2 I. U. of the same hormone together with an intravenous injection of 1 ml. of a 25 per cent solution of PVP (Table 4 B). A hundred per cent augmentation was found in the group receiving both hormone and PVP.

When the hormone was injected subcutaneously in a 25 per cent PVP solution together with 1 ml. of a 25 per cent PVP solution, injected intravenously, the augmentation was not higher than when no intravenous PVP injections was given.

Table 3.

Effect of varying the method of administration, and of injecting intravenously a 25 per cent solution of polyvinylpyrrolidone on the action of different doses of chorionic gonadotrophin.

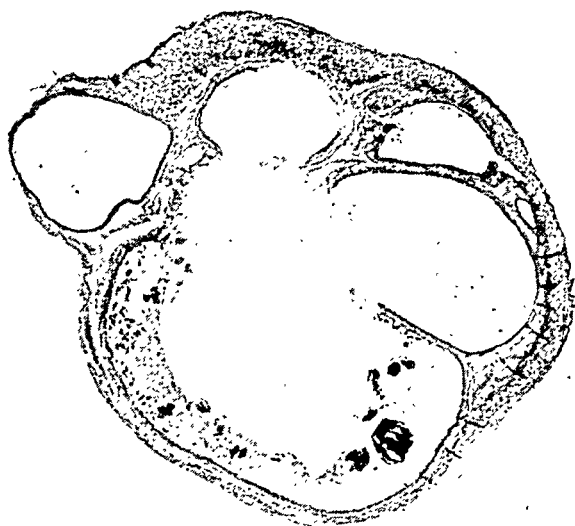
(Ten rats per dose.)

Mode of administration	Dose (in I. U.)	Per cent positive vaginal smears	Amounts of chor. gon. producing a 50 per cent posi- tive response
A. in saline intravenously	1.2	0	2.5
	2.5	60	
	5.8	100	
in PVP intravenously	0.8	0	1.6
	1.6	50	
	3.2	100	
B. in saline subcutaneously	0.6	0	1.6
	1.2	50	
	2.5	100	
in saline subcut. + 1 ml PVP intraven.	0.3	10	0.8
	0.6	50	
	1.2	100	
C. in PVP subcutaneously	0.15	0	0.3
	0.3	60	
	0.6	100	
in PVP subcut. + 1 ml PVP intraven.	0.15	0	0.3
	0.3	60	
	0.6	100	

### B. *Infantile Macacus rhesus*.

All the four monkeys were treated with 1000 I. U. PMS given as a single subcutaneous injection and five days later with 500 I. U. of chorionic gonadotrophin injection in the same manner. Two of the monkeys received the gonadotrophin in aqueous solution, the other two in a 25 per cent PVP solu-





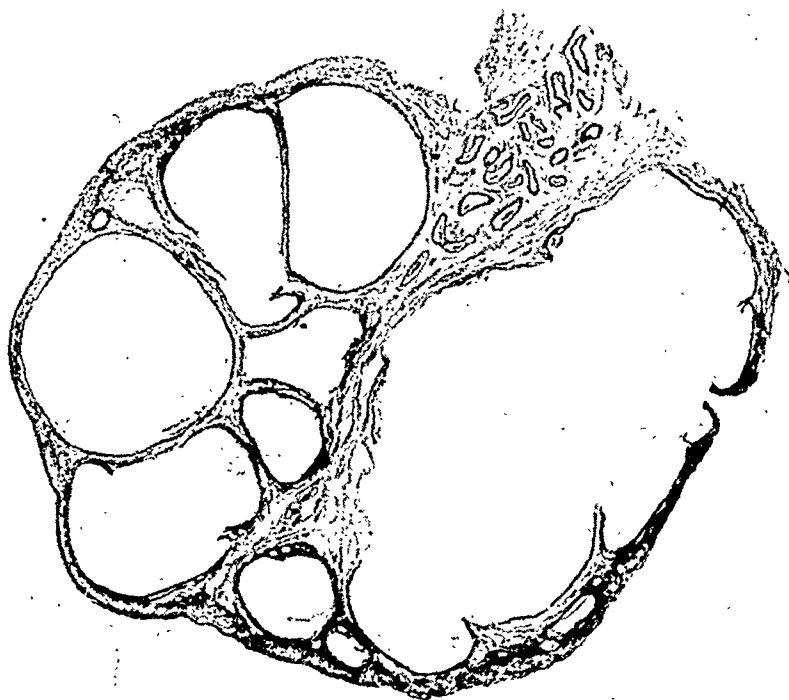
*Fig. 2* ( $\times 10$ ).

Section of an ovary from monkey 2 treated with 1000 I. U. mare serum gonadotrophin and 500 I. U. chorionic gonadotrophin dissolved in saline. The ovary consists chiefly of enlarged follicles among which an edematous stroma is seen containing two luteinized follicles of moderate size.

of the sexual skin from the fifth day of treatment. It progressed in a characteristic manner reaching its maximum at about the tenth day of treatment. Some days after castration a vaginal bleeding of 3—5 days' duration occurred in all 4 animals.

The weight of the ovaries is shown in Table 5. A remarkable augmentation of the ovarian response was obtained by using PVP solution instead of saline as solvent for the hormones. All ovaries were fixed in formalin and the sections were stained with hematoxylin-eosin.

The microscopic findings showed that the ovaries were largely composed of a few large follicles, of which some were luteinized. The results were essentially the same, whether the solutions had been given in aqueous or PVP solution (see Figs. 1—4).



*Fig. 3* ( $\times 10$ ).

Section of an ovary from monkey 1 treated with 1000 I. U. mare serum gonadotrophin and 500 I. U. chorionic gonadotrophin dissolved in polyvinylpyrrolidone, 25 per cent solution. The ovary contains several cystic follicles without any signs of luteinization.



*Fig. 4* ( $\times 10$ ).

Section of an ovary from monkey 4 treated with 1000 I. U. mare serum gonadotrophin and 500 I. U. chorionic gonadotrophin dissolved in saline. There is a moderate enlargement of some follicles in the cortical zone, but otherwise no signs of gonadotrophin stimulation.



## DISCUSSION

The finding that polyvinylpyrrolidone does not augment the gonadotrophic effect of pregnant mare serum hormone is in agreement with previous investigations in which it has been shown that the action of this gonadotrophin is not augmented by the addition of inert protein,  $\text{ZnSO}_4$ , etc., and with the fact that the effect of this substance is essentially independent of the mode of administration.

A remarkable augmentation of chorionic gonadotrophin is obtained when the hormone is dissolved in a 25 per cent solution of polyvinylpyrrolidone and given as a single subcutaneous injection. Solutions containing less than 25 per cent polyvinylpyrrolidone were less effective. It is reasonable to assume that the enhanced gonadotrophic effect is due mainly to a delayed absorption of the hormone. This delayed absorption does not seem to be the sole cause of the augmentation, as an augmentation was also observed when chorionic gonadotrophin was injected intravenously in combination with polyvinylpyrrolidone. The most reasonable explanation for these findings seems to be a delayed destruction and/or excretion of the hormone.

The finding, that no further augmentation was obtained when PVP was injected intravenously into rats injected subcutaneously with different doses of chorionic gonadotrophin and PVP, could be explained on the assumption that a maximal augmentation was already produced independently of the intravenous injection.

It is very likely that the addition of polyvinylpyrrolidone to chorionic gonadotrophin preparations will be of value in the clinical use of this hormones. Such experiments have been started and the results will be published later.

## SUMMARY

Polyvinylpyrrolidone which has been shown to delay the absorption of extracts from the posterior lobe of the hypophysis, was tried in combination with two commercial gonado-

trophin preparations, viz. pregnant mare serum gonadotrophin (Antex) and chorionic gonadotrophin (Physex).

In experiments with immature female rats, it was found that polyvinylpyrrolidone does not augment the effect of pregnant mare serum hormone, even when the hormone is dissolved in a 25 per cent solution of polyvinylpyrrolidone. On the other hand the effect of a subcutaneous injection of chorionic gonadotrophin is considerably augmented by polyvinylpyrrolidone. The augmentation is probably not entirely due to a delayed absorption, since an enhanced effect was also found when combinations of these substances were injected intravenously into immature rats.

Experiments with 4 immature female rhesus monkeys showed that in this species too the effect of gonadotrophic hormone on the ovarian weight is also considerably augmented by polyvinylpyrrolidone.

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